Characterization of Drosophila tumor-cell clones that bypass dependence on Ras for proliferation

Research Thesis

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By

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I. Abstract

Ras is a highly conserved gene in animals that is a key component of many different cellular pathways that control proliferation, differentiation, and cell death. In its oncogenic form, Ras is implicated in approximately 30% of human cancers. Our lab has generated Drosophila cell lines that express oncogenic Ras (Ras^{V12}) in an inducible manner. When Ras expression is turned on, the cells proliferate and when Ras is turned off, the cells stop proliferating. If the cells are maintained without RU486 for about three weeks rare cells start proliferating again and form colonies from which clonal lines were derived. These clones have bypassed dependence on Ras, presumably through genetic changes that activate other growth pathways (Fig. 1). Characterizing the Ras-bypass cells in relation to the parental lines is my thesis project. Seven different Ras-bypass clones were isolated. I conducted western blot analysis to characterize signaling pathways in these clones. The pathways I examined were ERK (MAPK), though which Ras signals, and the stress pathways involving AMPK and p38. From these data I determined that in contrast to the parental lines, the Ras bypass clones do not express stress proteins in the absence of Ras expression. In future work it will be important to determine the growth pathways that are activated in these cells that allow them to continue proliferating. In cancer, bypass mechanisms are often the reason tumors no longer respond to drug treatment targeting the original oncogene driver. In the long term, I expect my results may be useful for developing new therapies that overcome drug resistance by targeting additional pathways.

II. Introduction and Background

A. Introduction

Ras is a highly conserved gene that encodes a key downstream component of the epidermal growth factor pathway, which controls proliferation, differentiation, survival and cell growth (Downward, 2003; Wassarman, Therrien, & Rubin, 1995). Misregulation of this pathway can result in diseases like cancer. Cancer can be treated in multiple different ways, which include targeted therapy. However, the effectiveness of therapy is limited by drug resistance. Resistance can be acquired from a drug acting as a strong selector for a subpopulation of cells that are resistant to the drug (Holohan, van Schaeybroeck, Longley, & Johnston, 2013; Housman et al., 2014). These populations of cells have bypassed their dependence on the original oncogene and continue to proliferate because they have activated another pathway. It is important to understand how these bypass mechanisms work and to identify the pathways that allow cells to bypass dependency on the original oncogene. This information will be useful for developing new therapies that can target bypass pathways.

B. Studying bypass mechanisms in Drosophila

Our lab generated two Drosophila cells lines called Gene switch Ras (GSR2, GSR6), which express Ras^{V12} in an inducible manner. In these cells, Act5C-GeneSwitch-Gal4 regulates the expression of a UAS-Ras gene and a UAS-GFP reporter gene (Act5C is ubiquitously expressed). GeneSwitch-Gal4 is a variation of Gal4 and is only active when Mifepristone (RU486) is present. When RU486 is not present, the Gal4 protein is unable to activate the UAS-promoter and no transcription occurs (Duffy, 2002; Nicholson et al., 2008; Osterwalder, Yoon, White, & Keshishian, 2001). This system allows Ras to be induced only in the presence of the drug, RU486.

When the GSR cells are exposed to RU486, the cells express Ras and GFP. In the absence of RU486, the cells cease to express Ras and GFP and stop proliferating. Surprisingly, when Ras is not expressed for about three weeks rare cells begin to proliferate and form colonies. The clones have circumvented dependence on Ras, likely through genetic changes activating another pathway. This is analogous to how tumors circumvent dependence on an oncogene that a drug targets (Holohan et al., 2013; Husain et al., 2017; Niederst & Engelman, 2013). Seven different Ras-bypass clones have been isolated from two different parental cells lines by Amanda Simcox. I compared the Ras-bypass clones to the parental cells they are derived from to determine differences in gene expression and cell signaling.

The pathways I investigated are the ERK, p38, and AMPK pathways (Bradham & McClay, 2006; Cagnol & Chambard, 2010; Jeon & Hay, 2015; Loesch & Chen, 2008; Lusk, Lam, & Tolwinski, 2017; Olson & Hallahan, 2004; Roberts & Der, 2007; Wagner & Nebreda, 2009a, 2009b) (Wada et al., 2017). These pathways were shown to have differential expression in the parental cell lines, GSR2 and GSR6, depending on the presence or absence of RU486 (Ashley Rohrabaugh, a previous graduate student). I hypothesize, the Ras-bypass clones will show different regulation of these pathways. p38 and AMPK are stress pathways that are upregulated in response to cell stress such as oxidative stress, environmental stress and altered physiology of cells (Mihaylova & Shaw, 2011; ZARUBIN & HAN, 2005). To examine these pathways, I conducted western blot analysis on the seven Ras-bypass clones with (RU+) and without (RU-) RU drug.

III. Materials and Methods

A. Cell culture

GSR parental cells and Ras-bypass clones (GSR2-BP1, GSR2-BP2, GSR2-BP3, GSR6-BP1, GSR6-BP2, GSR6-BP3, and GSR6-BP4) were generated in the Simcox lab. These cells are cultured at 25°C in S2 Schneider's medium (Sigma) (supplemented with 10% Fetal Bovine Serum (Gibco, Life Technologies), and 1% Penicillin-Streptomycin solution (Gibco, Life Technologies). GSR cell lines were cultured with RU486 (Invitrogen) at a final concentration of 10nM to maintain proliferation. The Ras-bypass clones are no longer dependent on RU486 and are cultured in the absence of RU486. Cells were harvested in accordance to standard procedures (appendix - Protocol).

B. Western Blot Analysis

Western blots were conducted in accordance to standard procedures (appendix Protocol). Primary antibodies were used at the following dilutions 1:1000 Erk (Cell Signaling Technologies), concentration of 1:1000 dpERK (Cell Signaling Technologies), 1:1000 AMPK (Cell Signaling Technologies), 1:1000 pAMPK (Cell Signaling Technologies), 1:5000 α-tubulin (Cell Signaling Technologies), 1:1000 p38 (Santa Crus Biotechnologies), 1:1000 pp38 (Santa Crus Biotechnologies), and 1:2500 Ras (Oncogene Research Products). Secondary antibodies were used at the following dilutions 1:2000 anti-rabbit (Cell Signaling Technologies) 1:5000 anti-mouse (Cell Signaling Technologies), and 1:1000 anti-goat (ICN biomedicals). Relative protein expression was estimated by normalization to α-tubulin.

IV. Results and Discussion

A. Ras-bypass clones express Ras in the presence of RU486 indicating the cells have an intact GeneSwitch-Gal4-UAS system

Western blots were conducted on the parental cells and the seven Ras-bypass clones cultured with and without RU486, which induces Ras expression. This allowed me to determine if the Ras-bypass cells still induced Ras expression in the presence of RU486 and therefore had an intact GeneSwitch-Gal4-UAS system. The parental cells express Ras in the presence of RU486. The Ras-bypass also clones expressed Ras in response to RU486 (Fig. 2) This shows that Ras is still being regulated by the GeneSwtich-Gal4-UAS system and suggest that Ras^{V12} (and GFP, Fig. 3, Fig. 4) are not expressed unless activated by Act5C-GeneSwitch-Gal4.

I examined expression of dpERK (activated MAPK), which is downstream of Ras. In the parental cells, dpERK levels are elevated slightly when Ras^{V12} is expressed. However, in GSR2-BP2, GSR6-BP1, GSR6-BP2, and GSR6-BP4 Ras-bypass clones there was no change in dpERK expression with or without Ras expression (Fig. 2). A previous graduate student who initially characterized these cells also found no significant variation in dpERK expression with or without Ras expression in the parental lines. I do not have definitive data for GSR2-BP1, GSR2-BP3 and GSR6-BP3, which have large standard deviations, and more replicates are required (Fig. 5, Fig. 6). GSR2-BP2, GSR6-BP1, GSR6-BP2, and GSR6-BP4 accumulate less dpERK than the parental cells. This suggests that there is another pathway that is activating dpERK.

Both parental and Ras-bypass clones express GFP and Ras when RU486 is introduced to the media. Thus, GFP is used as a reporter for UAS-transgene expression in cells. In parental cells, GFP is expressed in all cells when RU is present. This is the case for all Ras-bypass clones except one (appendix Cell Images). In the Ras-bypass colony GSR6-BP2, only a small number of cells

express GFP. These express GFP with and without RU drug, although the GFP signal is brighter with RU drug.

B. Ras-bypass clones do not exhibit stress signaling in the absence of Ras expression

In the parental cells, pAMPK is upregulated when Ras is not expressed, this is likely due to stress induced by altered physiology of the cells. However, in GSR2-BP1, GSR2-BP2, GSR2-BP3, GSR6-BP1, GSR6-BP3, and GSR6-BP4 Ras-bypass clones pAMPK levels do not correlate in the same way as the parental lines with Ras expression (Fig. 2). There is one exception — in GSR6-BP2, however, more replicates are required, as the standard deviation is large. The other Ras-bypass clones expressed lower levels of pAMPK than the parental cells in the absence of RU486 (Fig. 7, Fig. 8). This suggests that the Ras-bypass cells experienced lower stress in the absence of Ras^{V12} expression than the parental cells.

In the parental cells, p38 is upregulated when Ras is not expressed. In GSR2-BP1, GSR2-BP2, GSR6-BP1, GSR6-BP2, GSR6-BP3, and GSR6-BP4 Ras-bypass clones, p38 expression is not significantly different with or without Ras expression (Fig. 2). GSR2-BP3 cells expressed more p38 with Ras expression, however more replicates are required as the standard deviation is large. Ras-bypass clones express lower levels of p38 than the parental cells in the absence of RU 486 (Fig. 9, Fig. 10). This suggests that Ras-bypass cells experienced lower stress in the absence of Ras^{V12}than the parental cells.

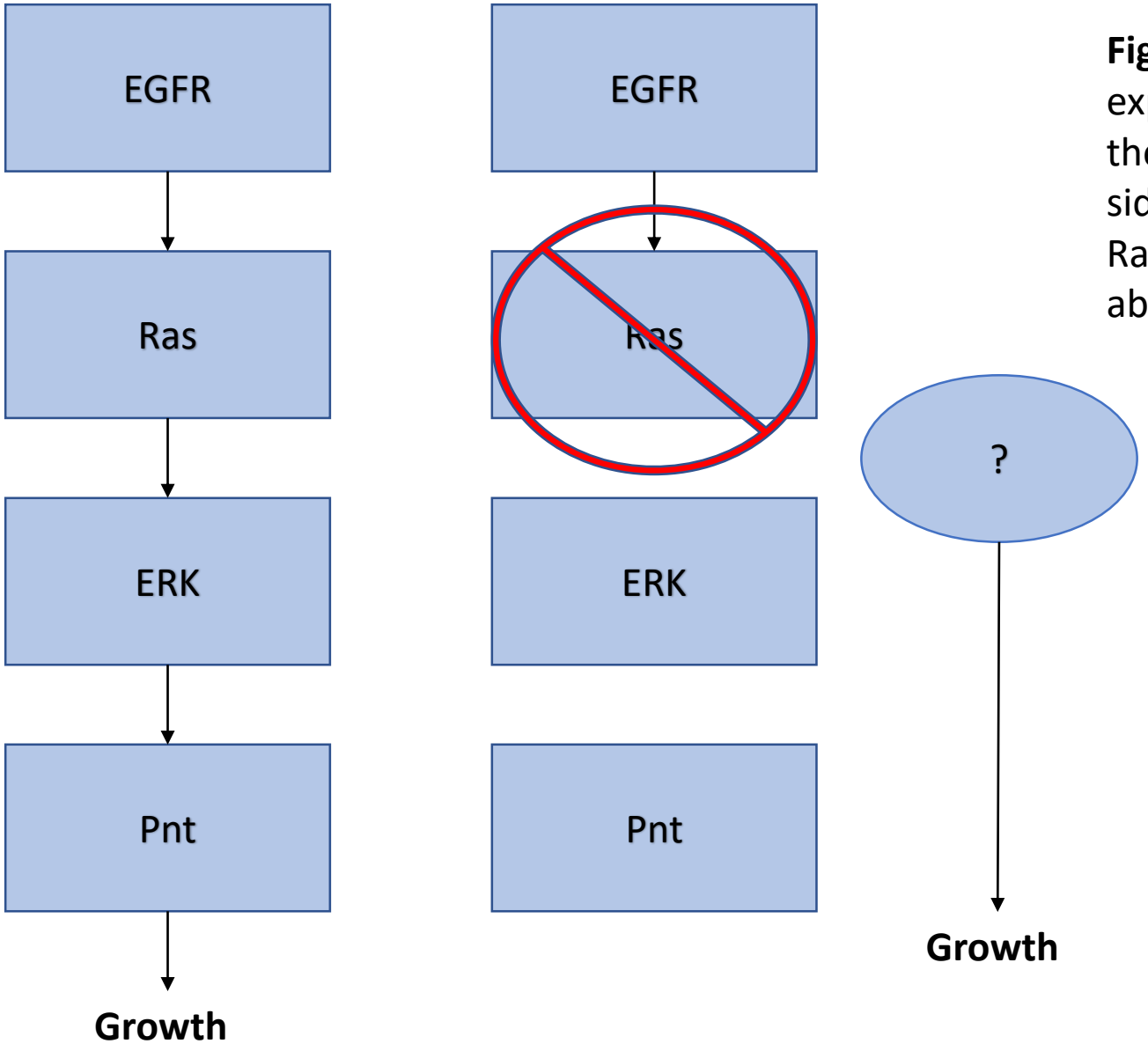

Figure 1: Left side depicts expression in GSR cells in the presence of RU. Right side depicts expression in Ras-bypass clones in absence of RU.

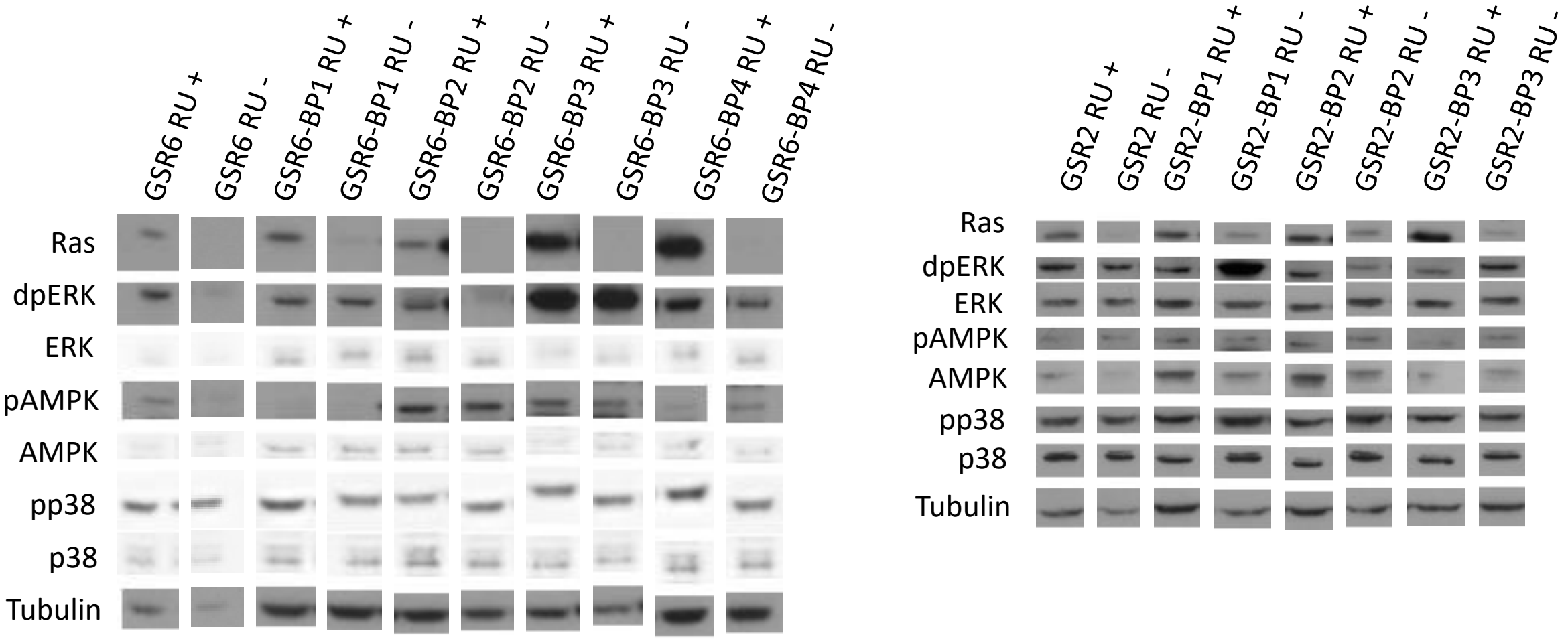

Figure 2: Representative western blots (set 2) for GSR2 and GSR6 derived Ras-bypass colonies grown in the presence and absence of RU486. RU + indicating presence RU486 and RU- indicating absence of RU486.

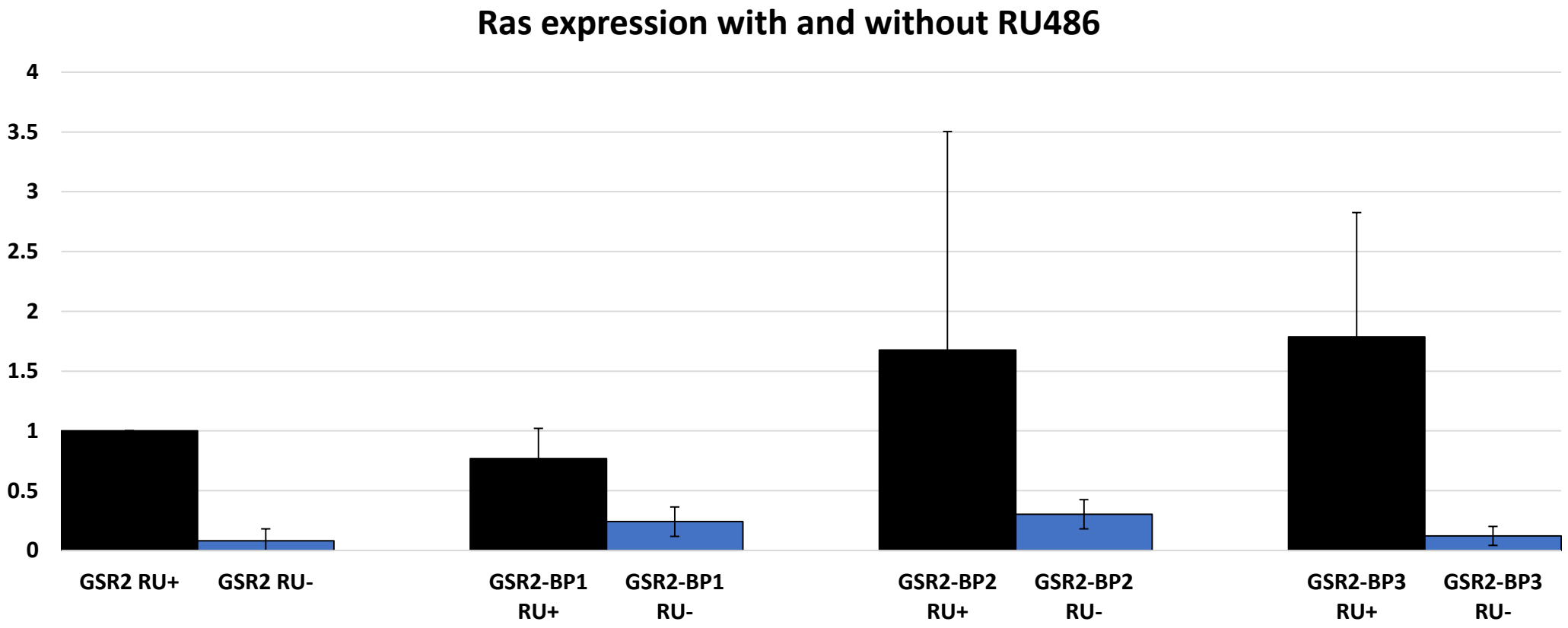

Figure 3: The graphs show Ras expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR2 samples n=4) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

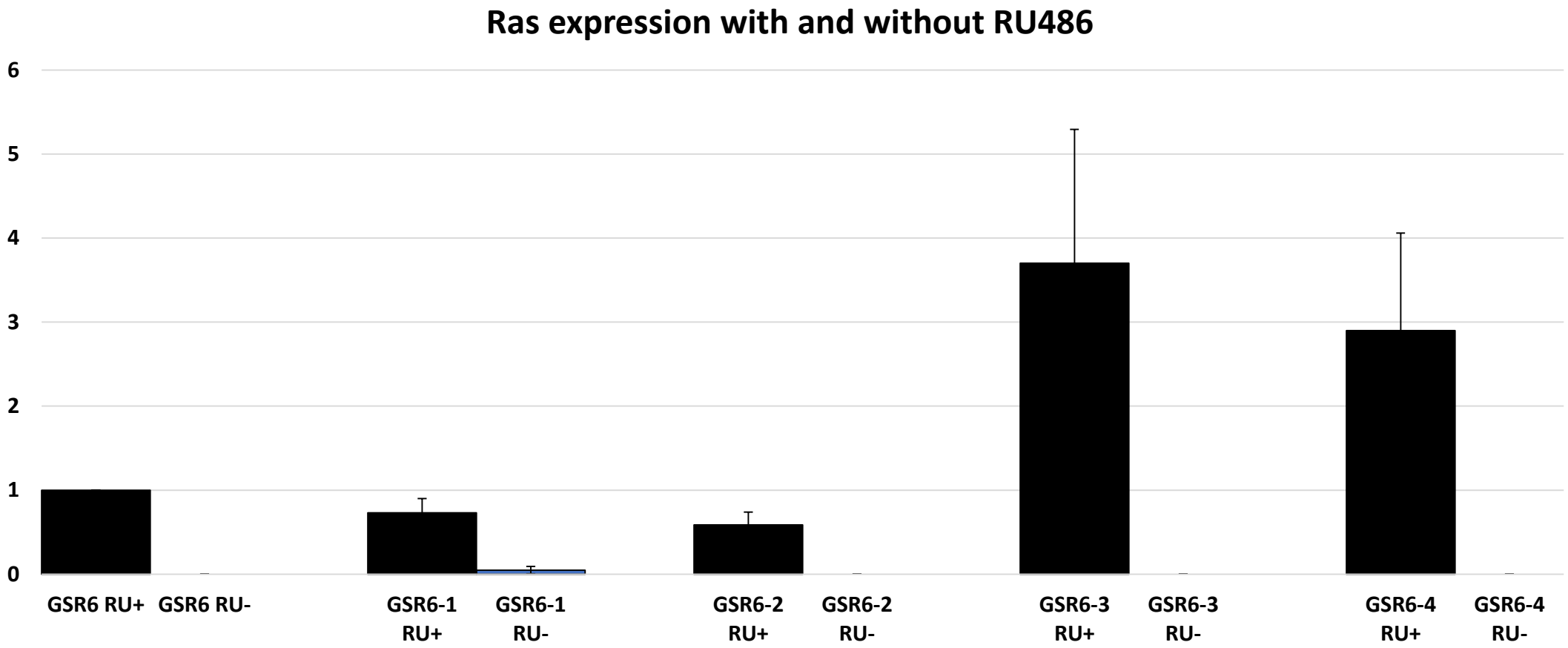

Figure 4: The graphs show Ras expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR6 samples n=3) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

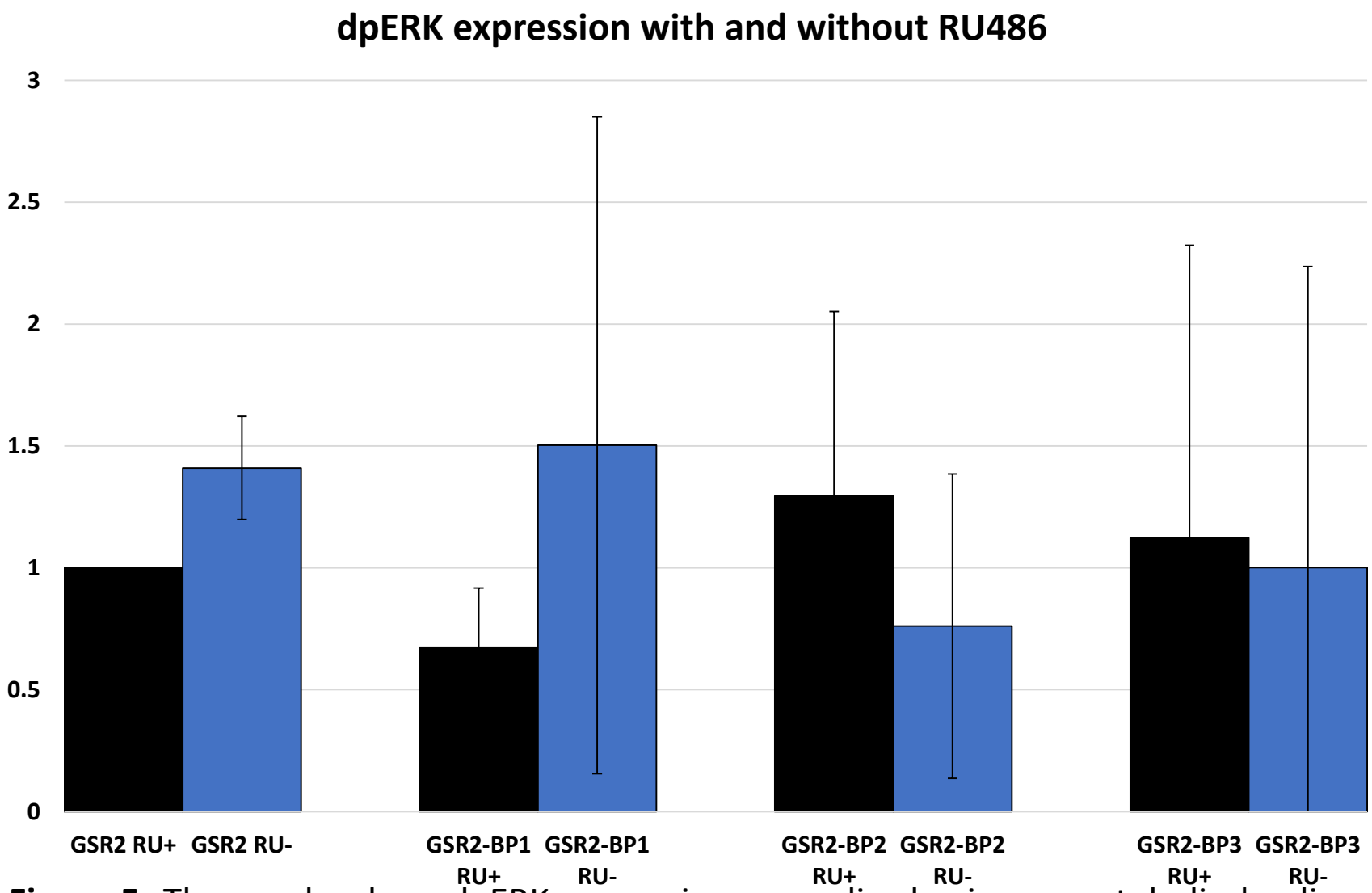

Figure 5: The graphs show dpERK expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR2 samples n=4) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

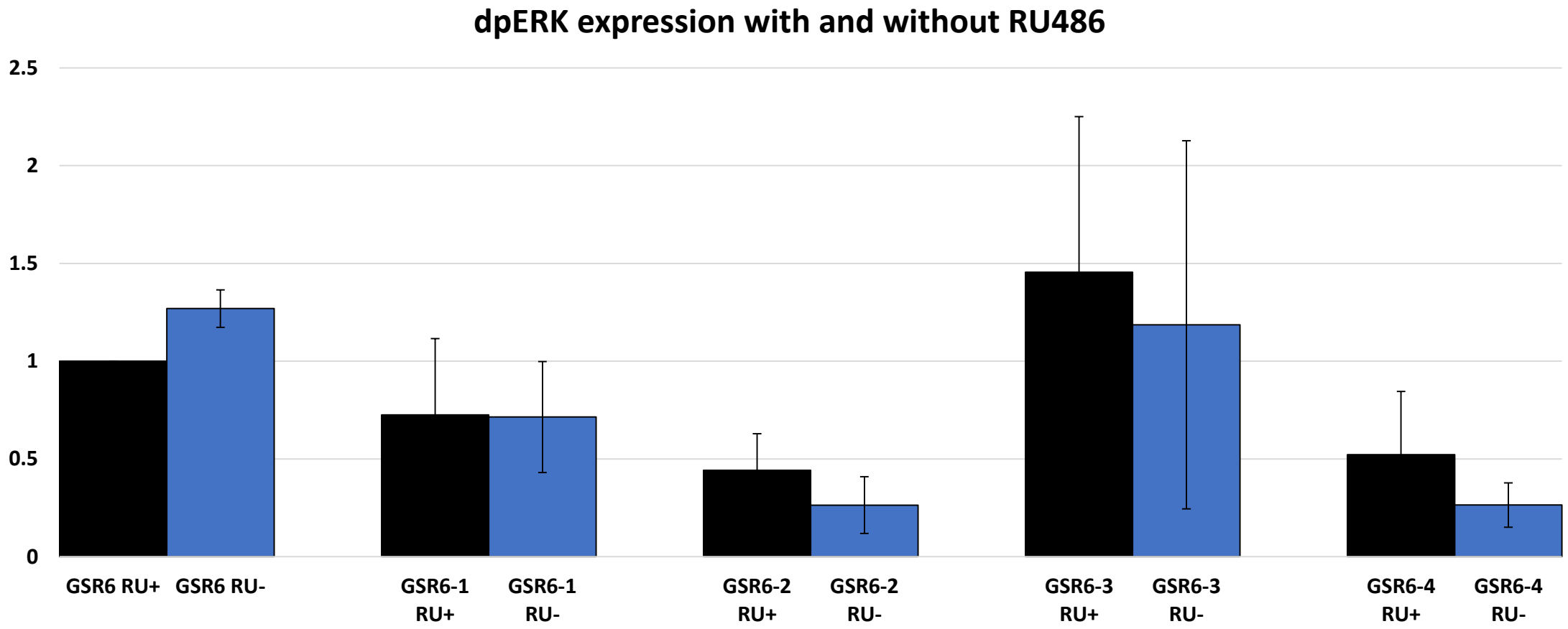

Figure 6: The graphs show dpERK expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR6 samples n=3) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

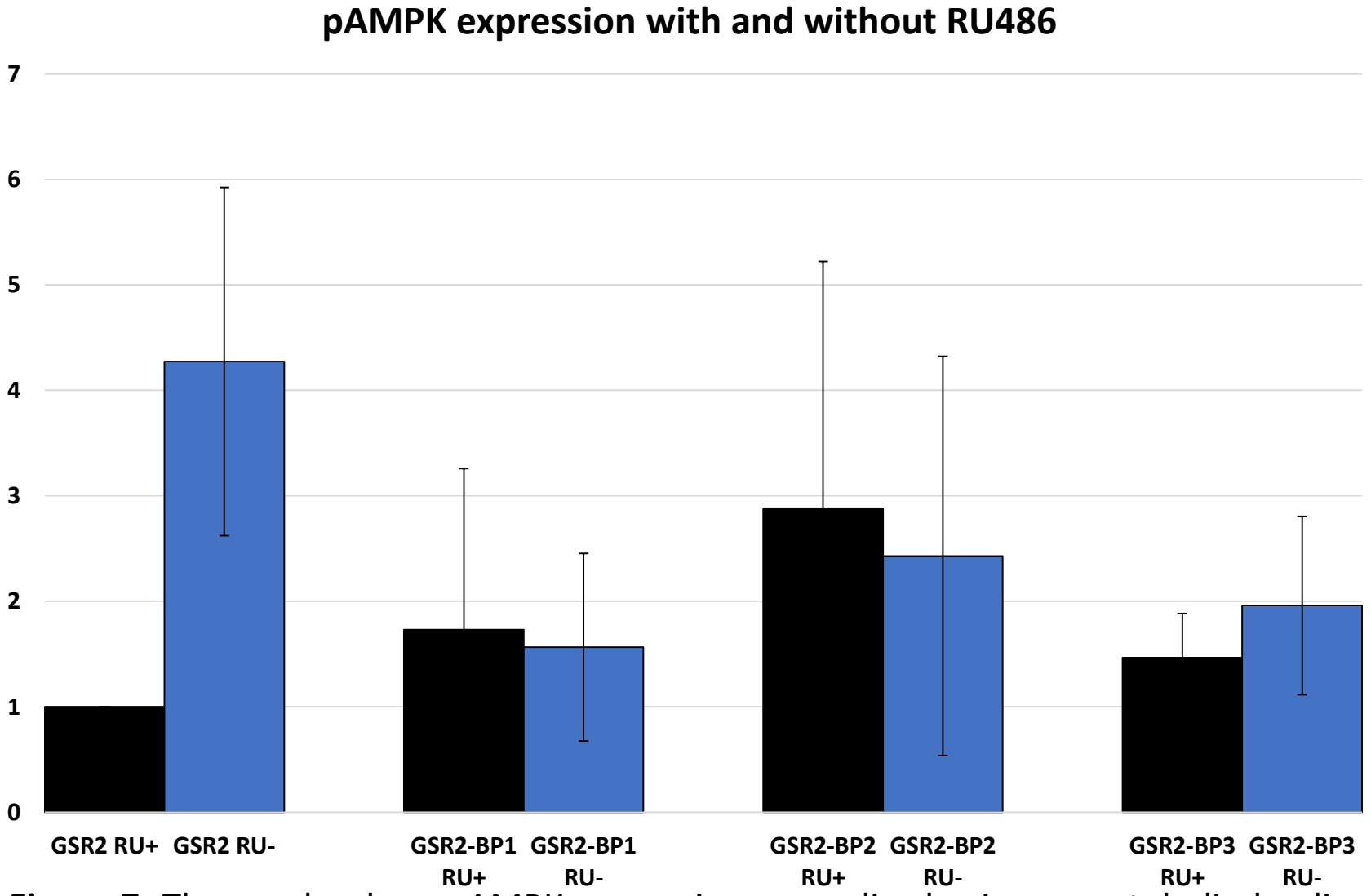

Figure 7: The graphs show pAMPK expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR2 samples n=4) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

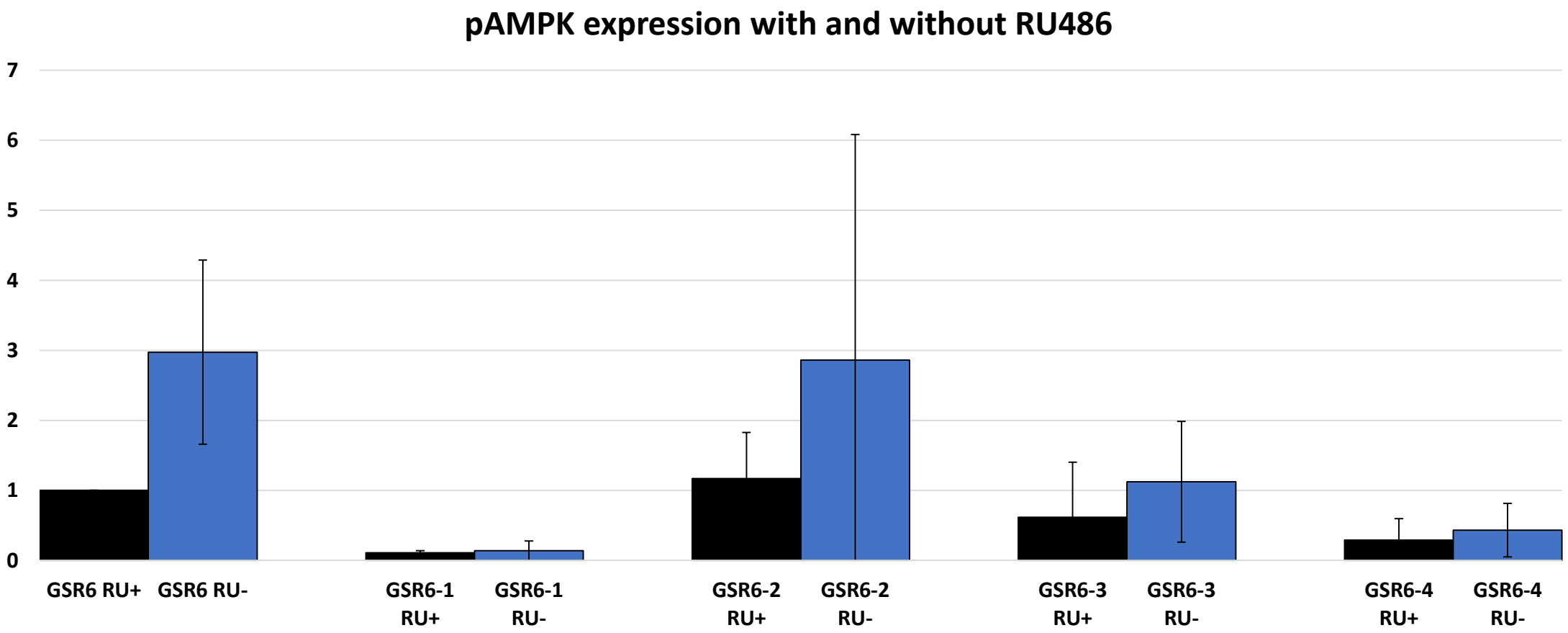

Figure 8: The graphs show pAMPK expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR6 samples n=3) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

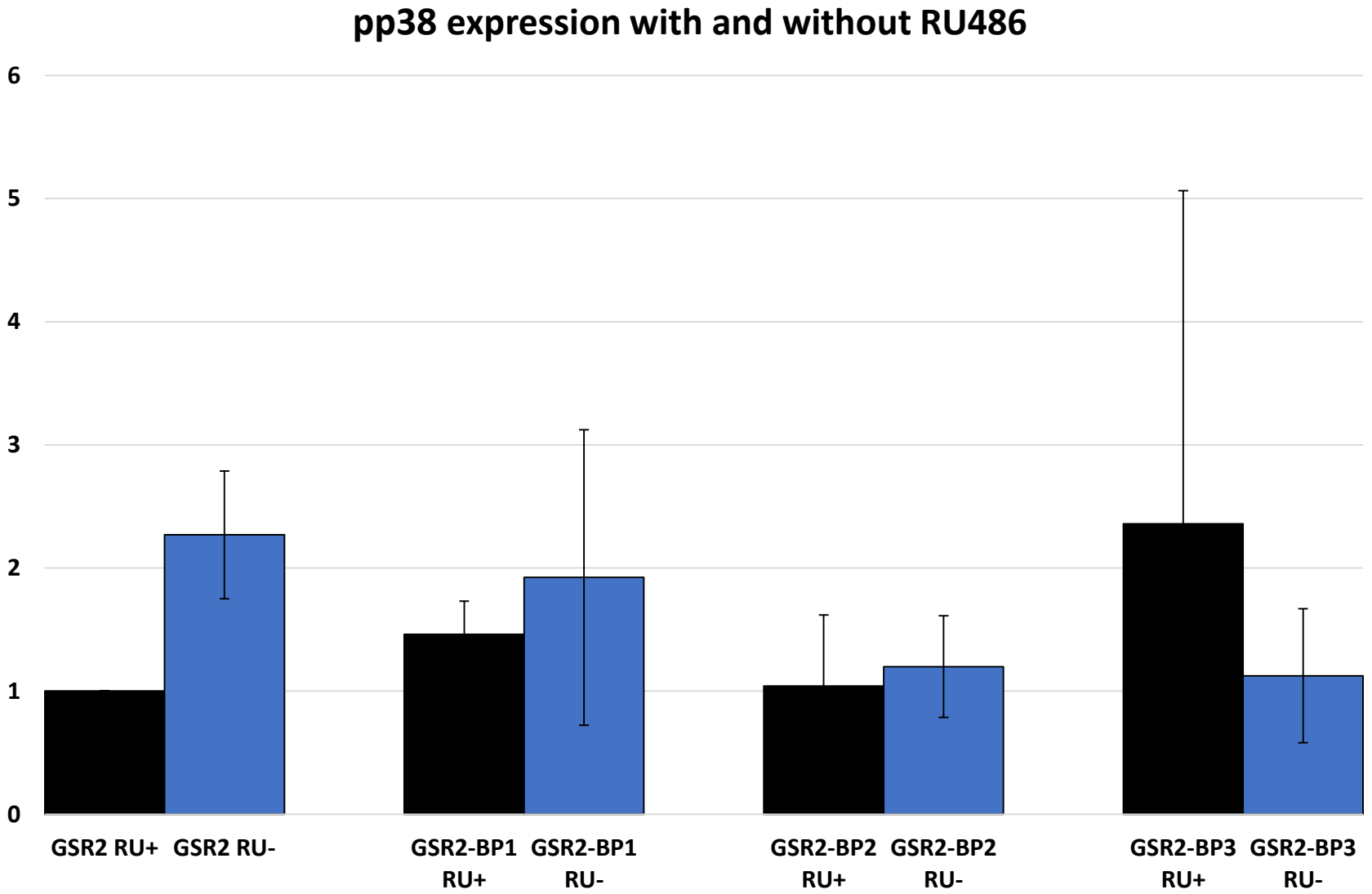

Figure 9: The graphs show pp38 expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR2 samples n=4) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

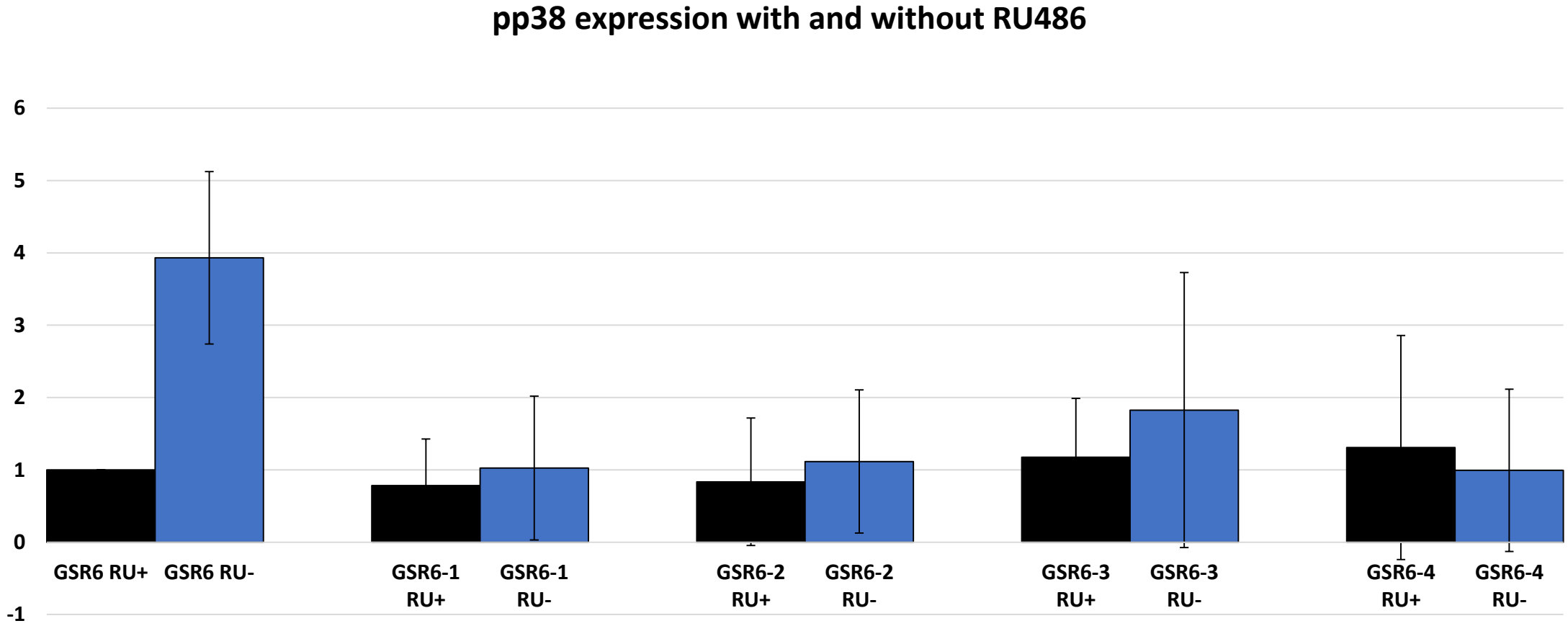

Figure 10: The graphs show pp38 expression normalized using an α-tubulin loading control. Columns show average expression and error bars show standard deviation (GSR6 samples n=3) RU+ means the colony has been grown in the presence of the RU486 drug and RU- means no RU486 was present.

V. Acknowledgements

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VI. References

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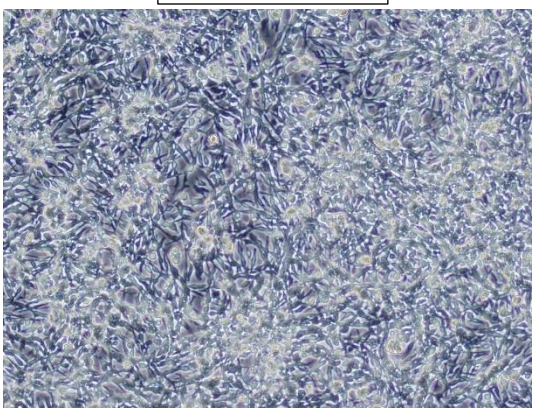
VII. Appendix – Cell Images

Both parental and Ras-bypass clones express GFP and Ras when RU486 is introduced to the media. Thus, GFP is used as a visual marker for Ras expression in cells. In parental cells, GFP is expressed in all cells when RU is present. This is the case for all Ras-bypass clones except one. In the Ras-bypass colony GSR6-2, only a small number of cells express GFP. In these cells GFP is expressed in a small number of cells both with and without RU drug, although the GFP signal is brighter with RU drug. This suggest that only a small number of cells are expressing Ras. It is possible that a mutation has altered the expression of GFP in these cells.

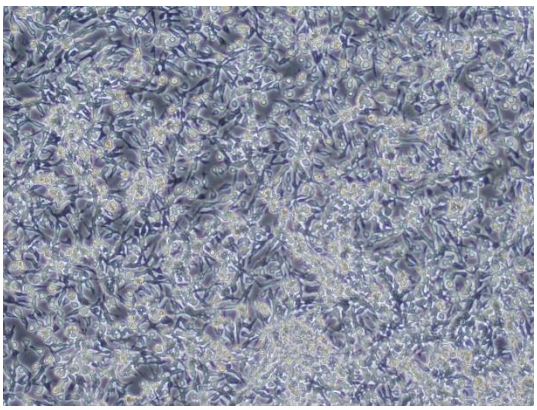
The Ras-bypass clones have different morphologies than the parental cells. Cells will also change morphologies depending on Ras expression. In the parental cells expressing Ras, cells are circular/ovular and have visible and distinguishable cell outlines. In parental cells without Ras expression, the cells are smaller compared to their RU+ counterparts. In contrast, the Ras-bypass cells have similar morphologies with and without the drug. All cell images are in the appendix.

GSR2 set 1

GSR2-BP1 RU +



GSR2-BP1 RU -



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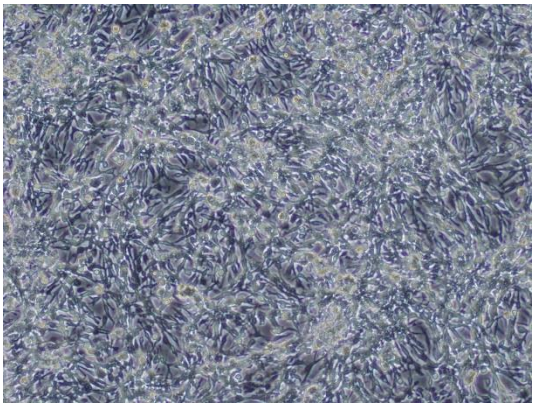

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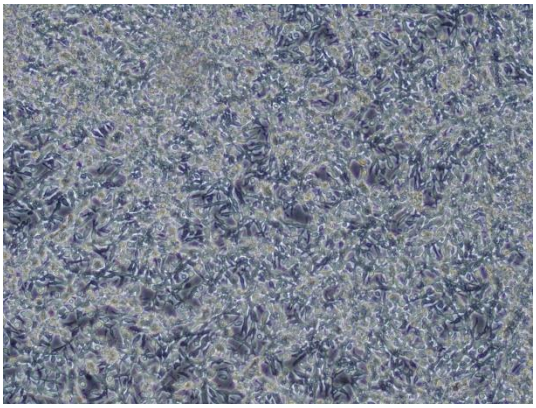
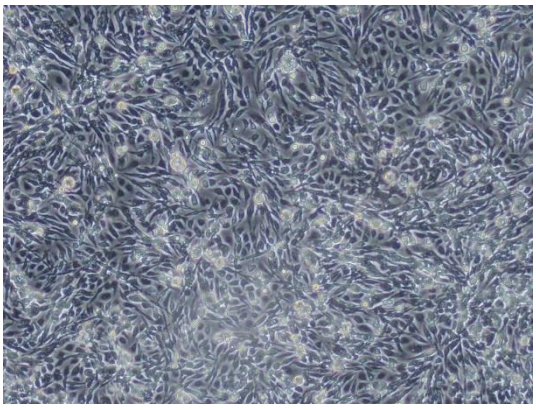


GSR2-BP3 RU +



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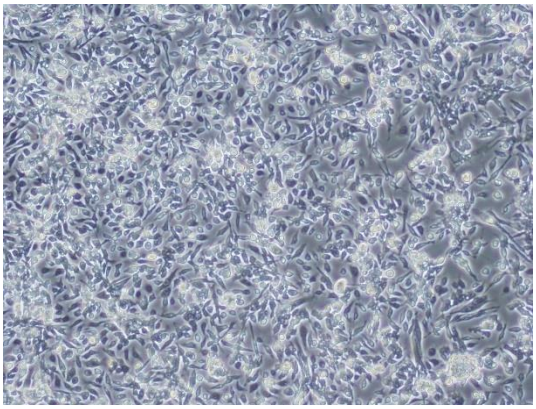
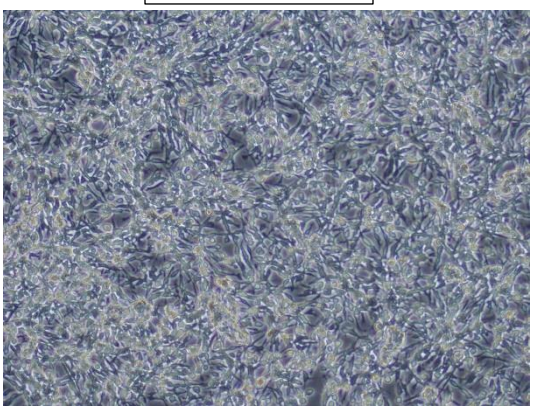


GSR2 set 2

GSR2-BP1 RU +



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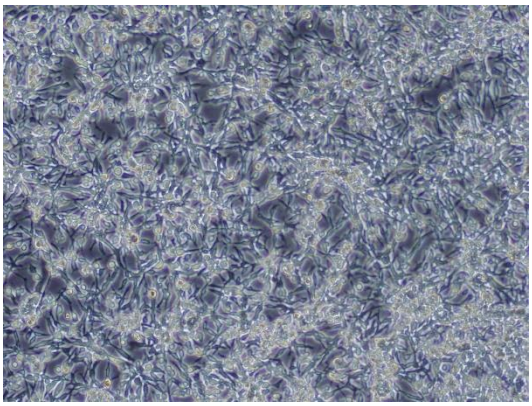

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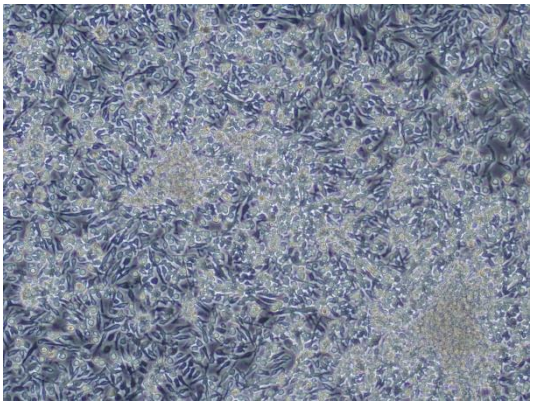

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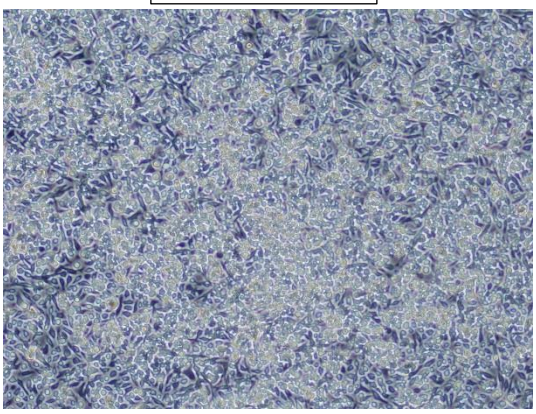
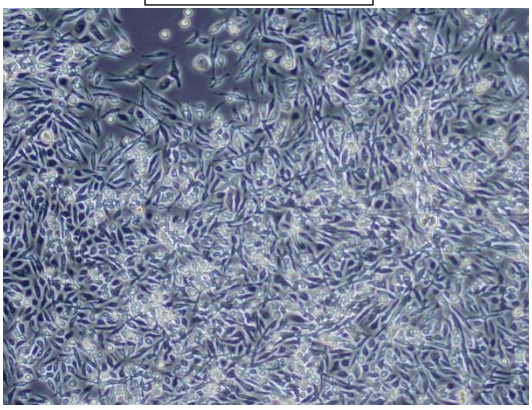
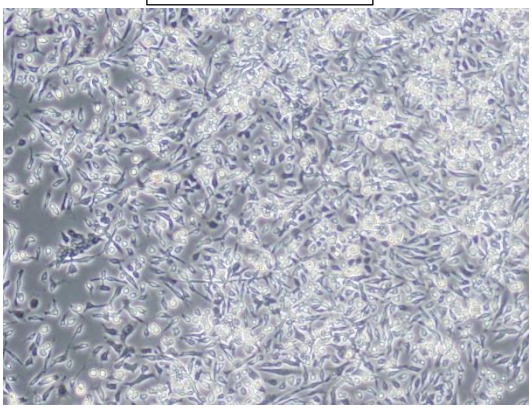


GSR2-BP3 RU +

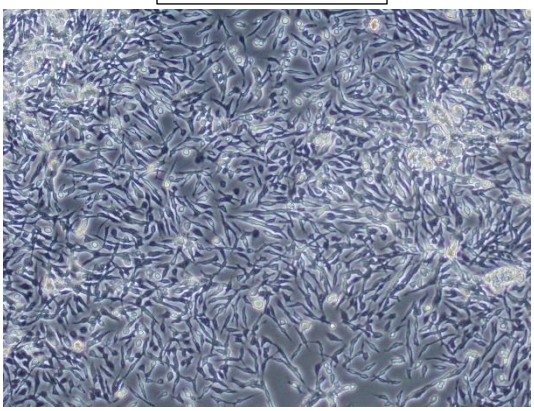


GSR2-BP3 RU -



GSR2 set 3

GSR2-BP1 RU +



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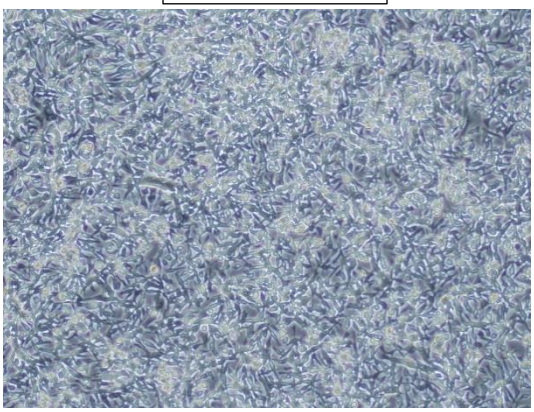

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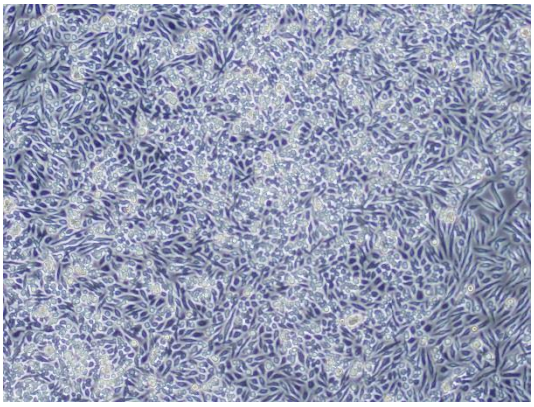

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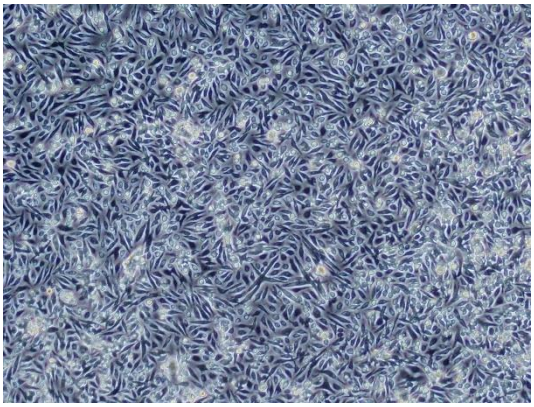

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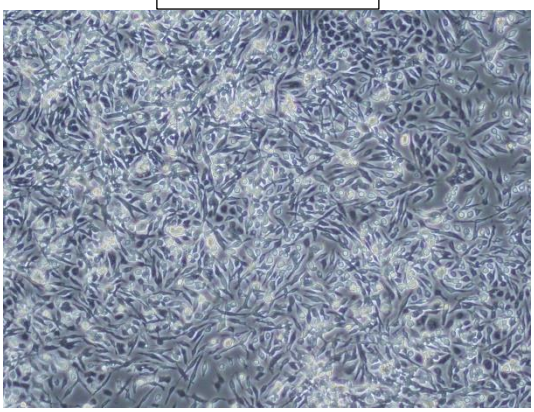

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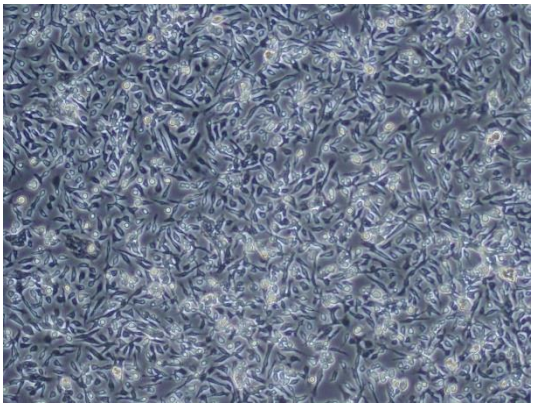
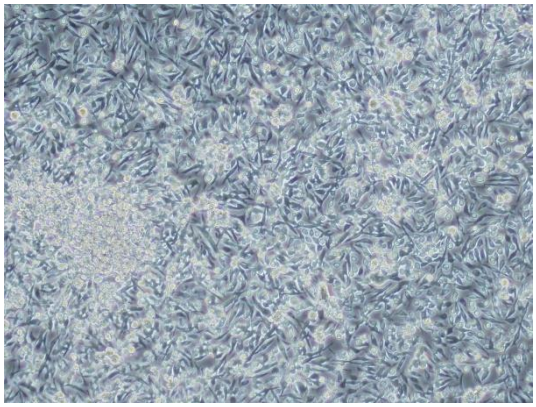


GSR2 set 4

GSR2-BP1 RU +



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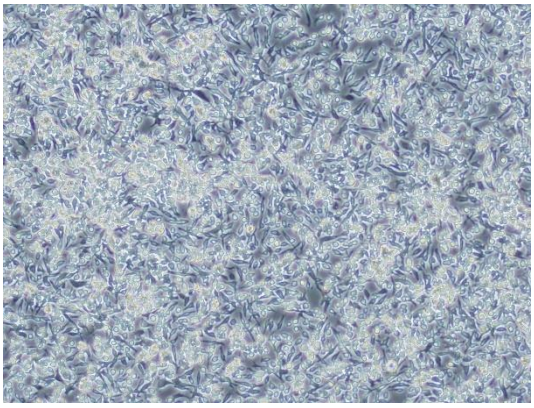

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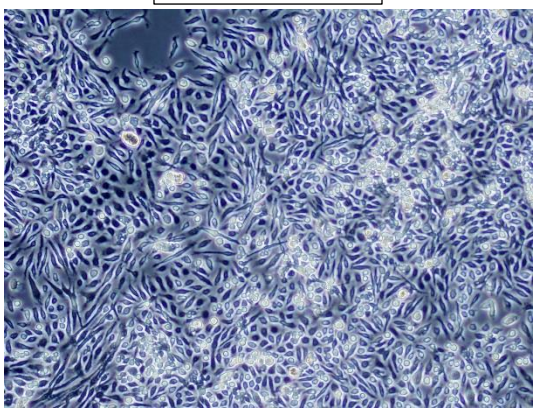

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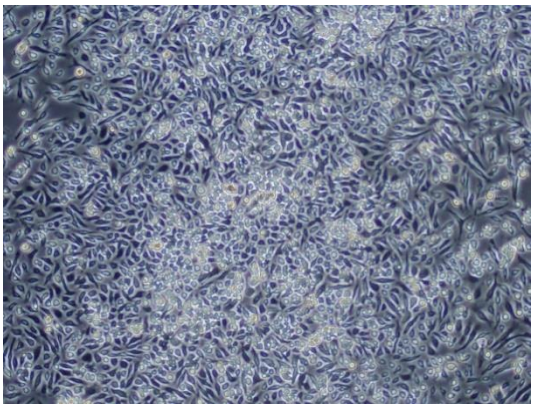

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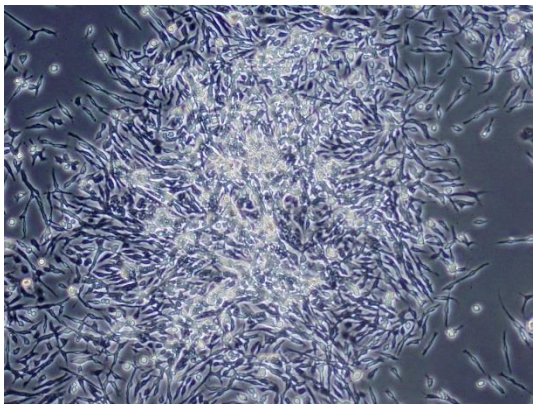

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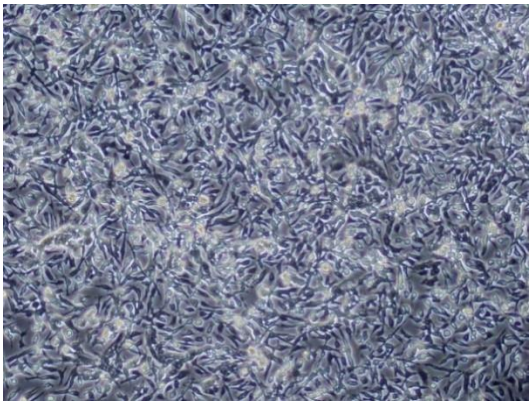

GSR2 set 1 GFP

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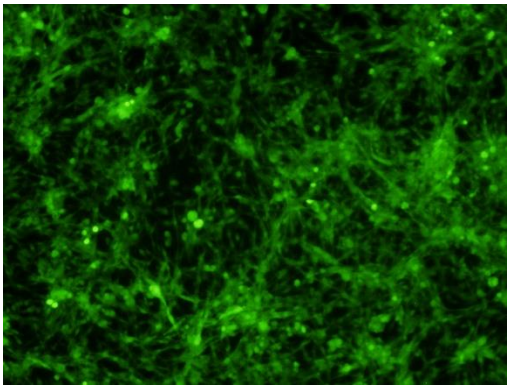
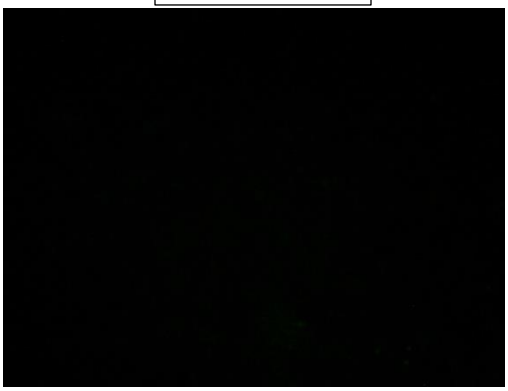


GSR2-BP1 RU -



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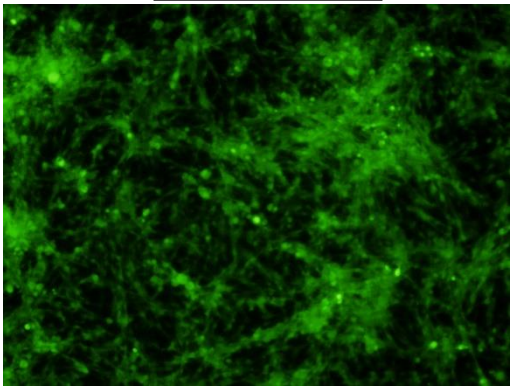

GSR2-BP2 RU -

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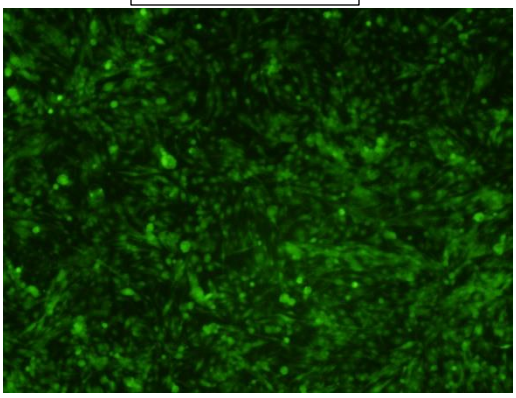

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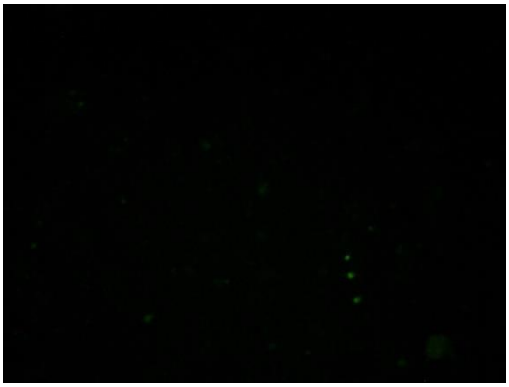
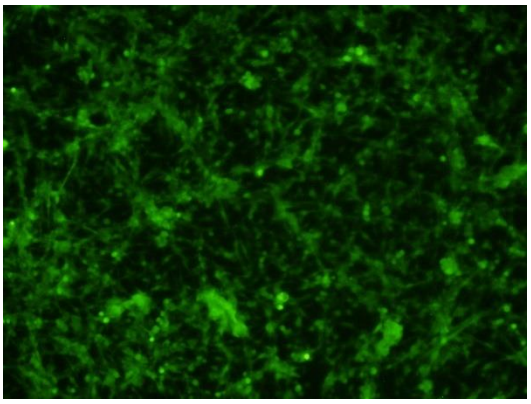


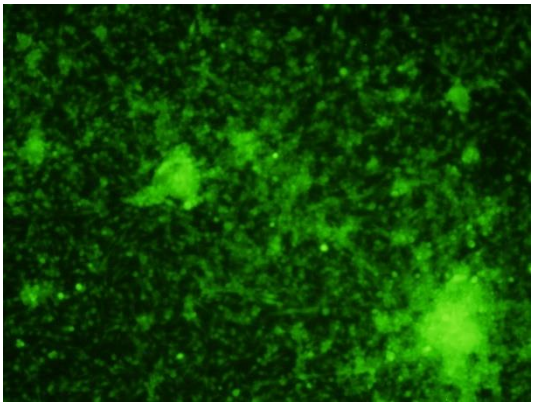
GSR2 set 2 GFP

GSR2-BP1 RU +



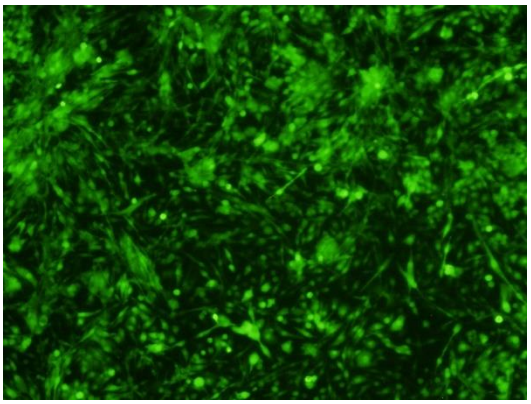
GSR2-BP1 RU -

GSR2-BP2 RU +



GSR2-BP2 RU -

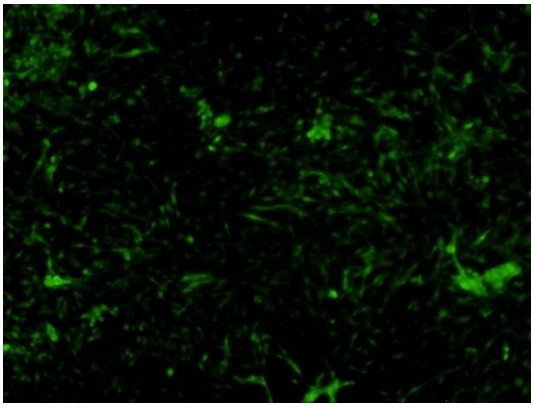
GSR2-BP3 RU +



GSR2-BP3 RU -

GSR2 set 3 GFP

GSR2-BP1 RU+



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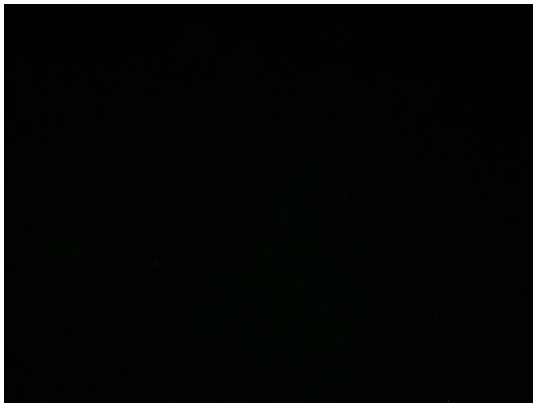

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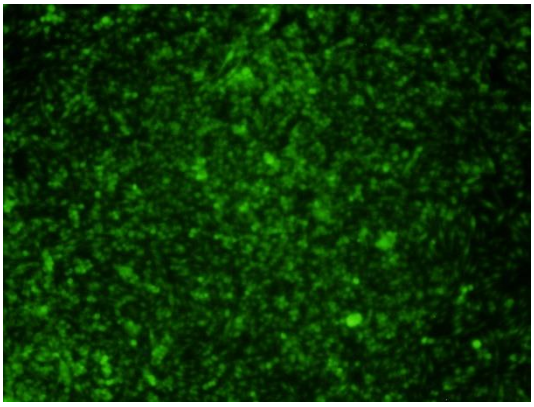

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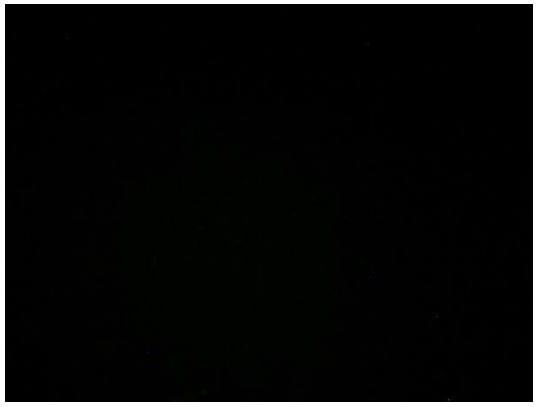

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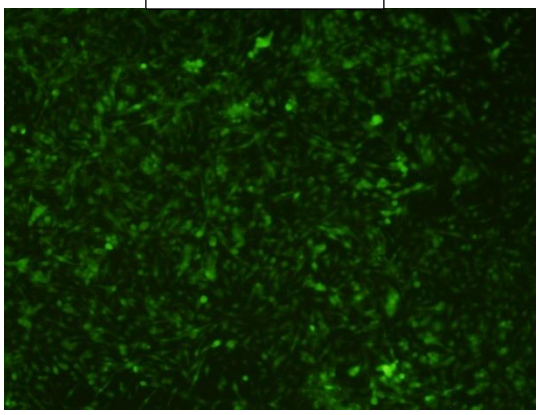
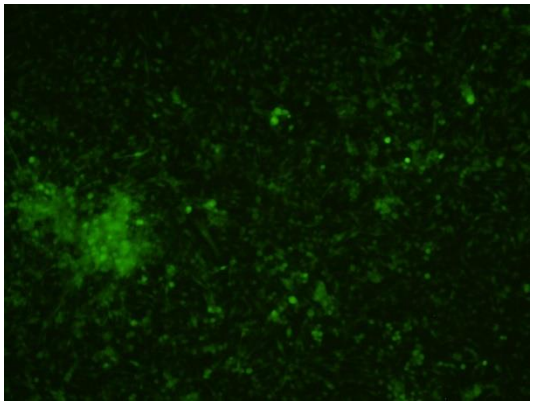


GSR2-BP3 RU -

GSR2 set 4 GFP

GSR2-BP1 RU +



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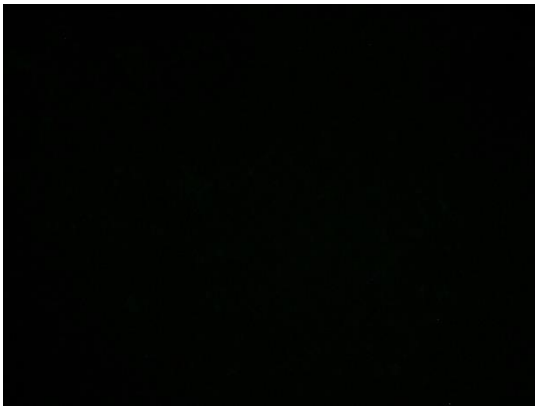

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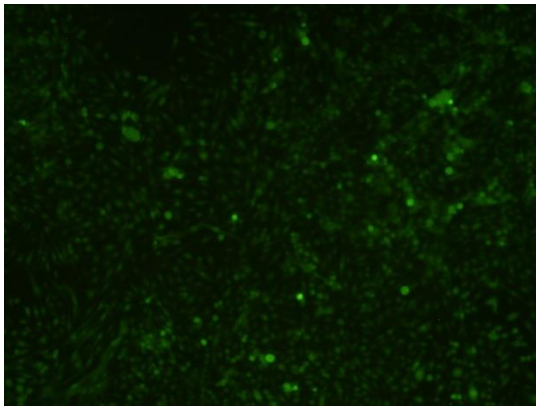
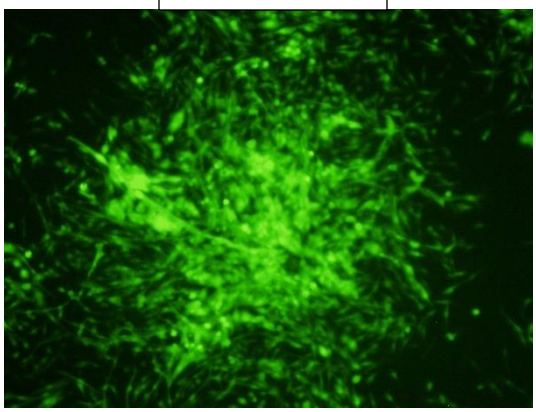


GSR2-BP2 RU -

GSR2-BP3 RU +



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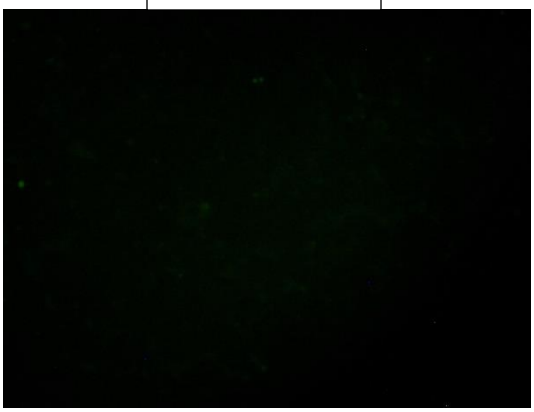
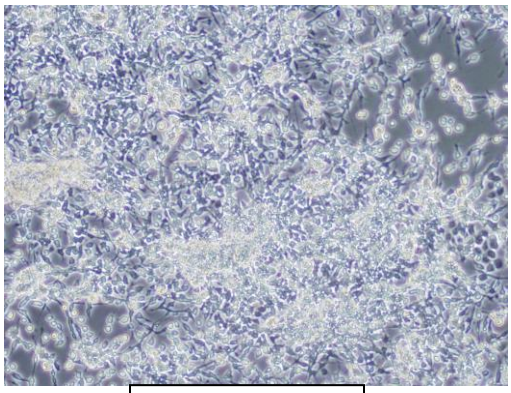


GSR6 set 1

GSR6-BP1 RU +



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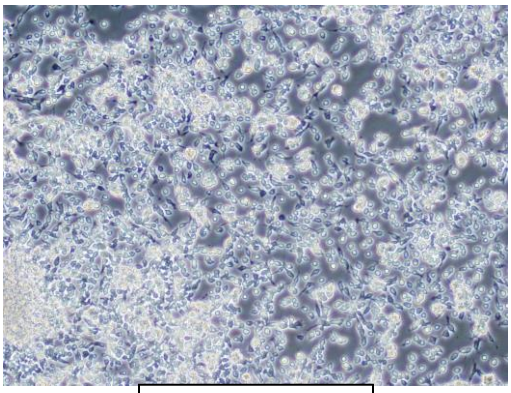

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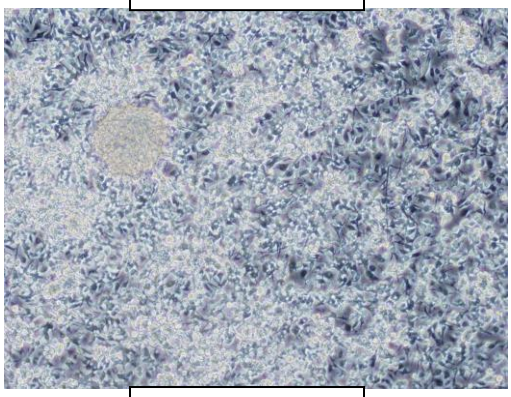

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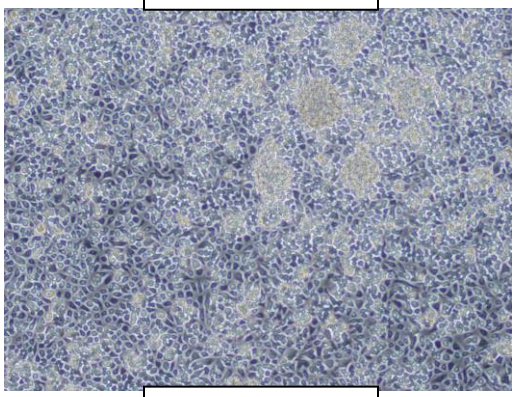

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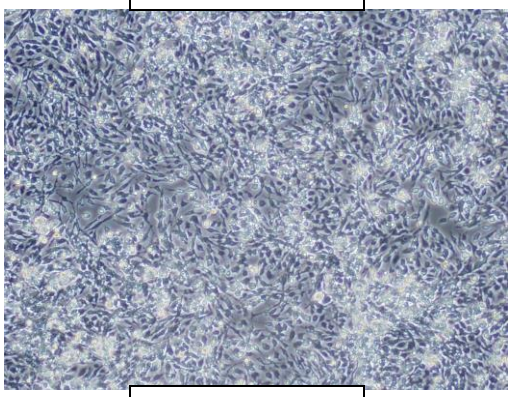

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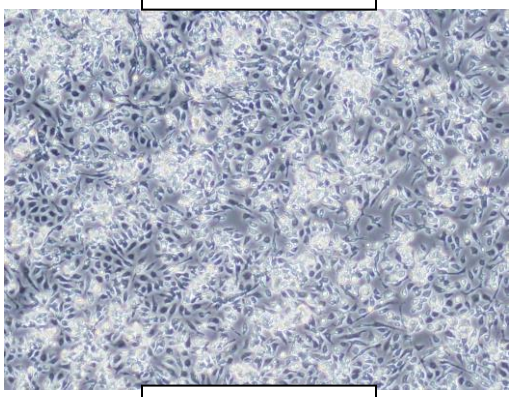
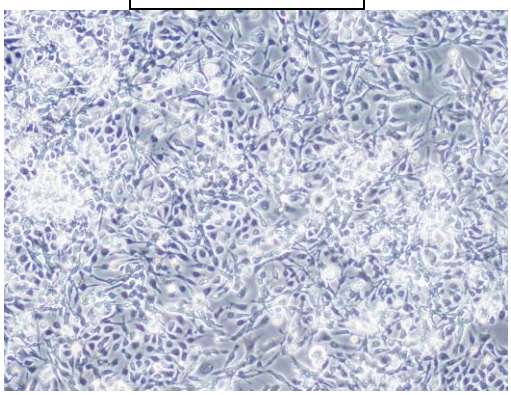


GSR6-BP4 RU +



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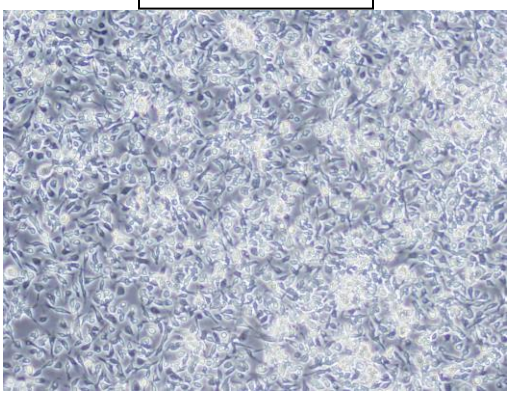
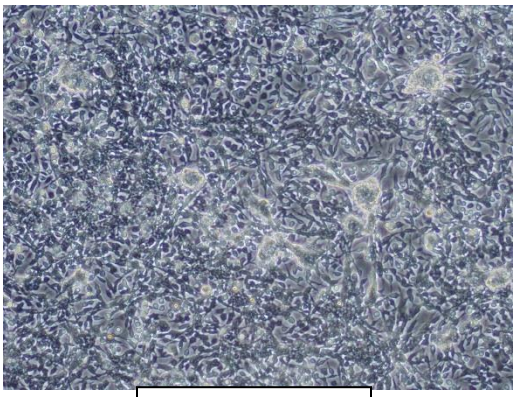
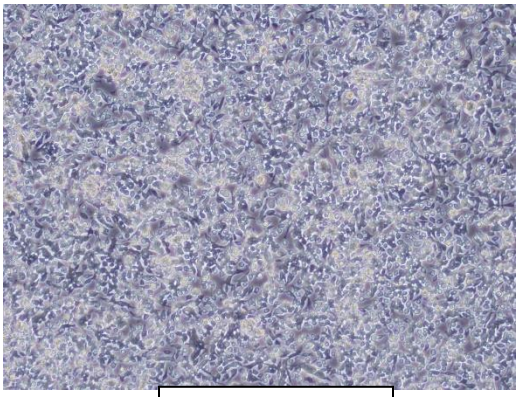


GSR6 set 2

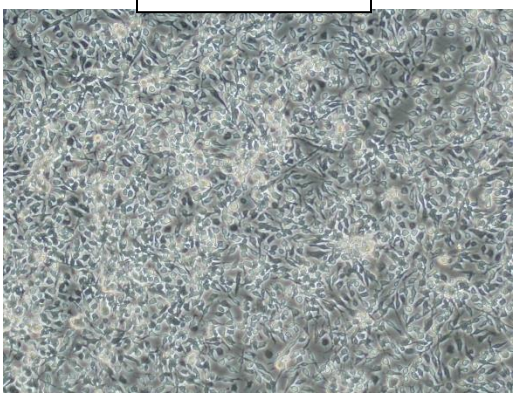
GSR6-BP1 RU +



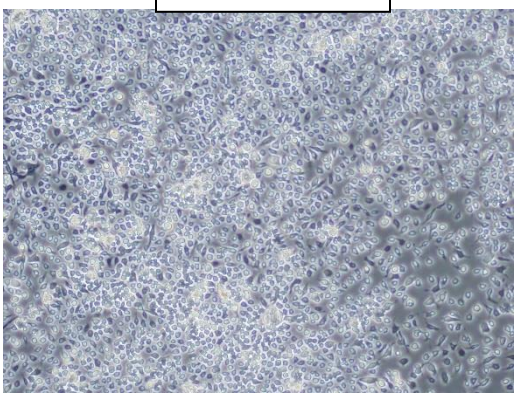
GSR6-BP1 RU -



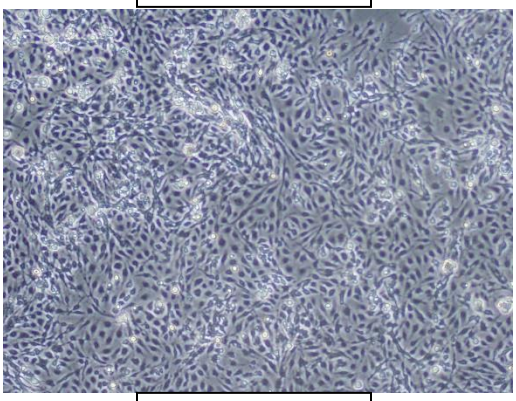
GSR6-BP2 RU +



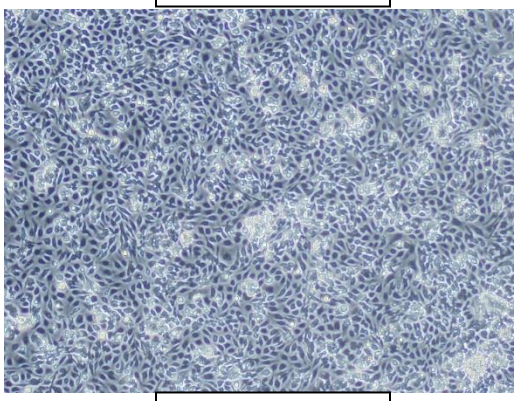
GSR6-BP2 RU -



GSR6-BP3 RU +



GSR6-BP3 RU -



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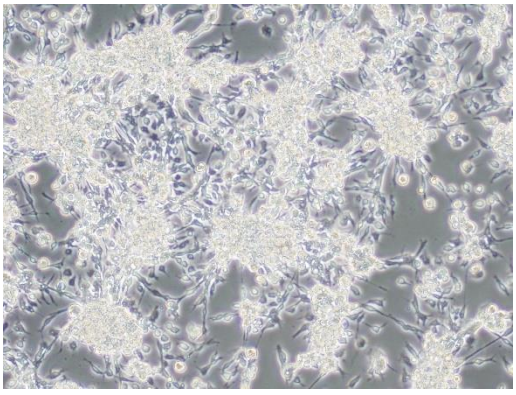

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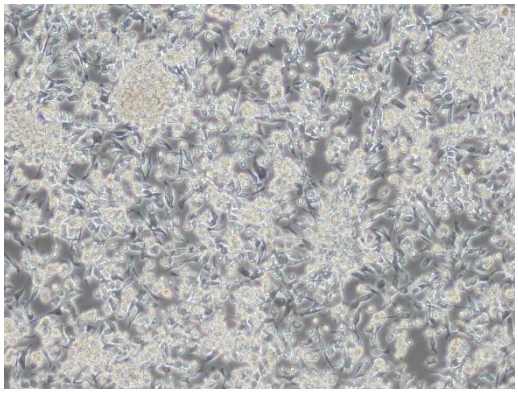
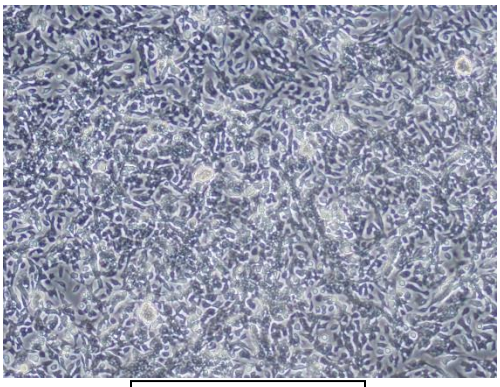


GSR6 set 3

GSR6-BP1 RU +



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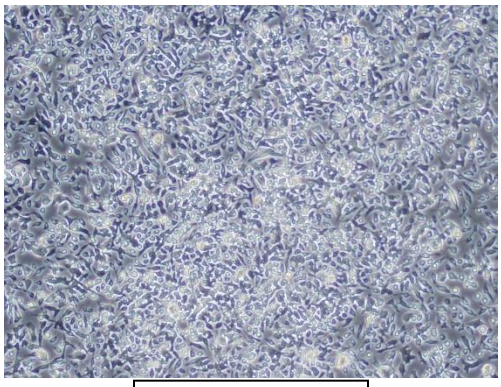

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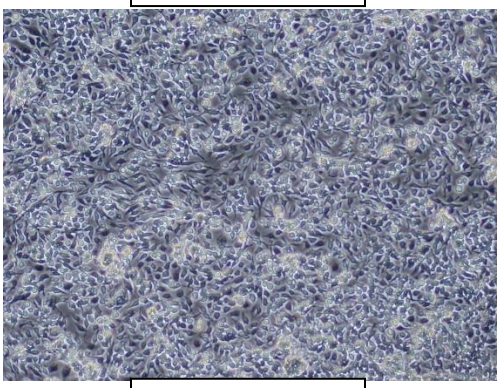

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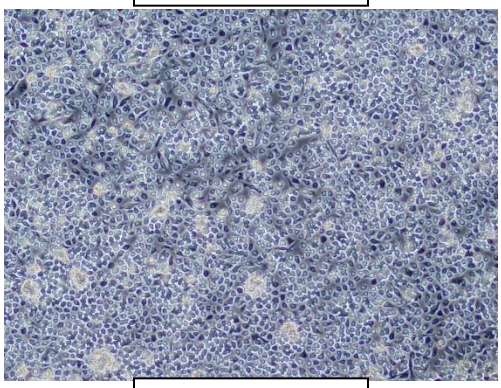

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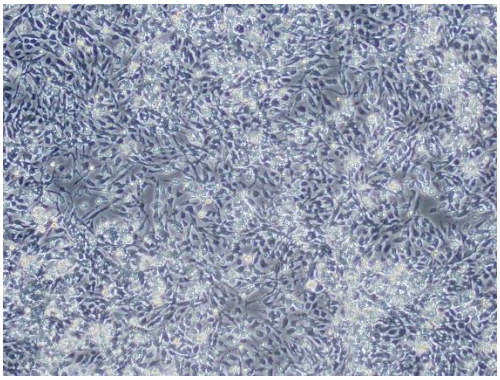

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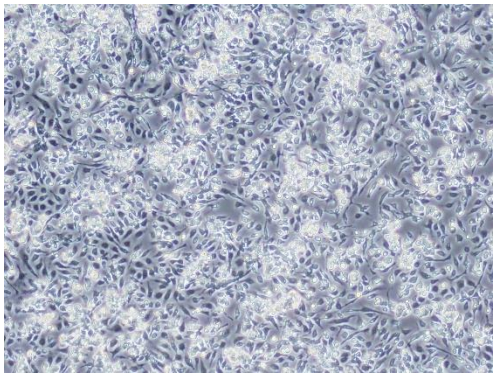

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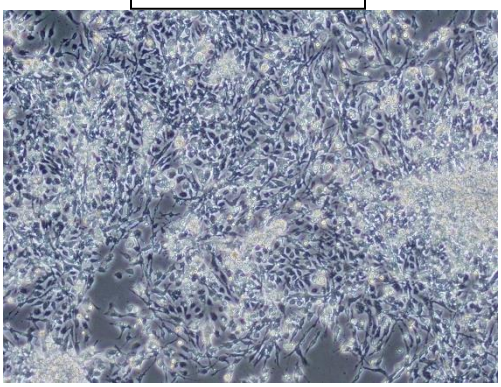

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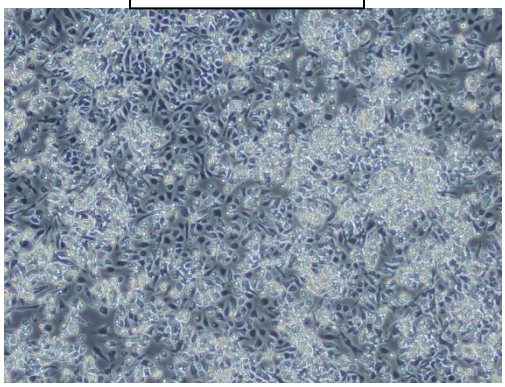

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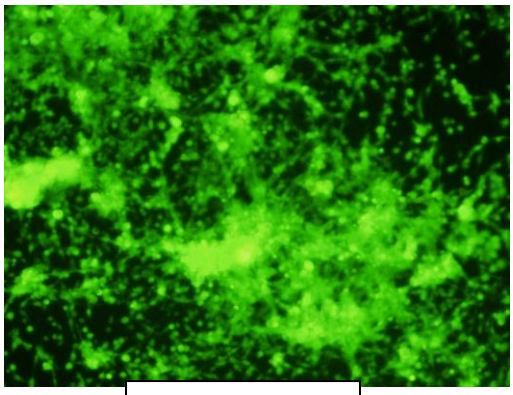

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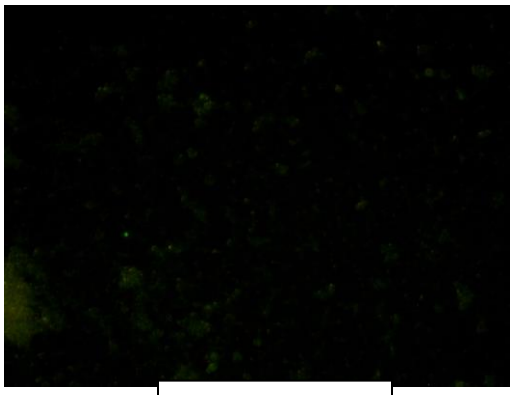

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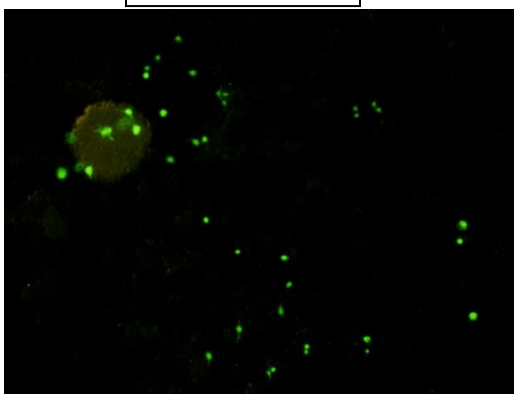

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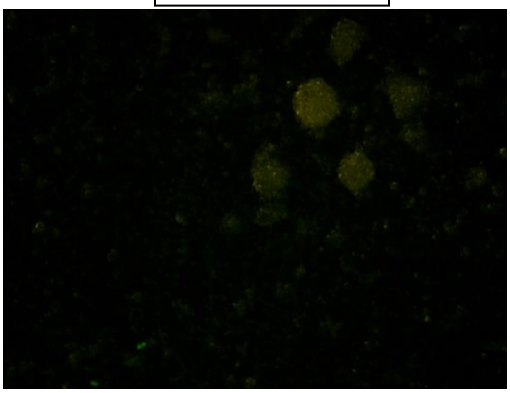

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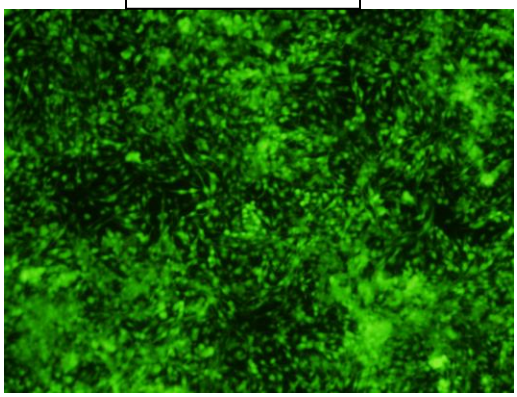

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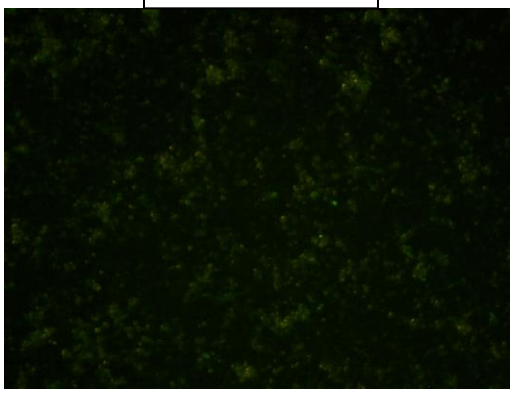

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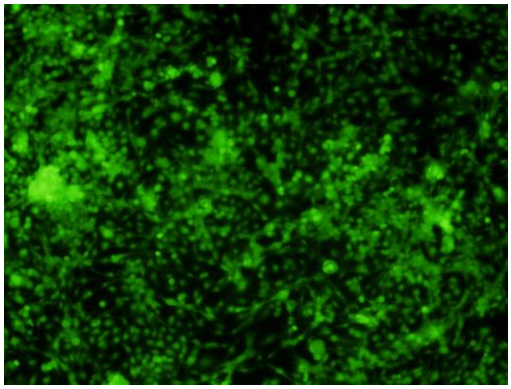

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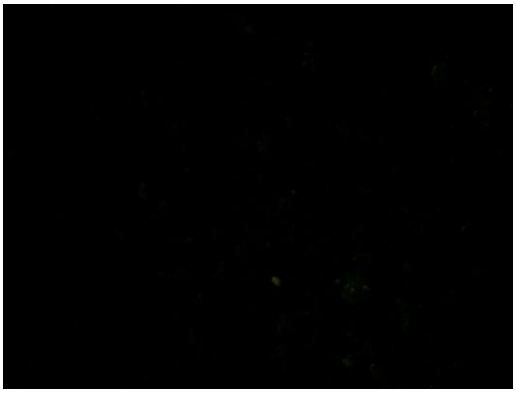

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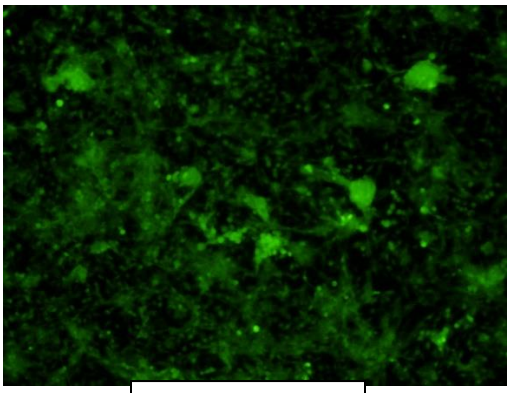

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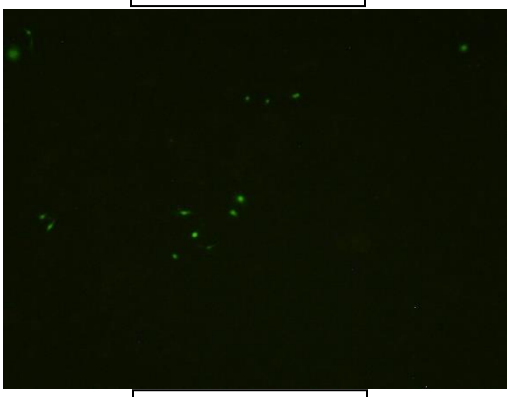

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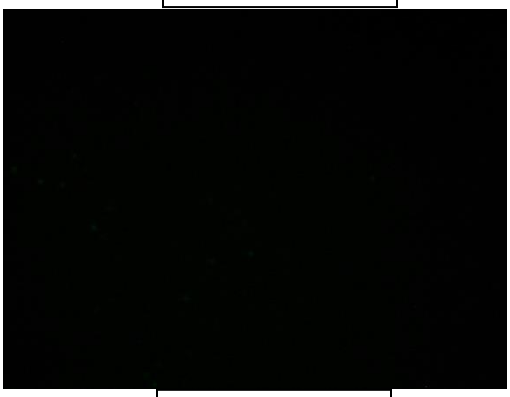

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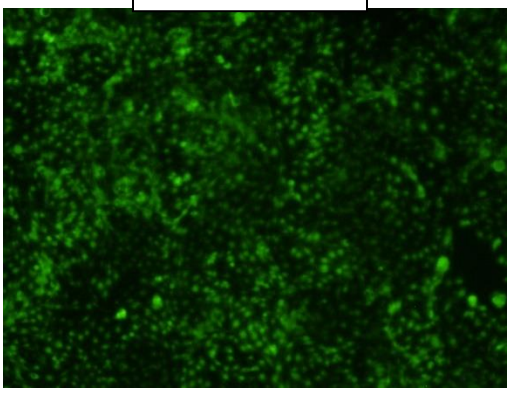

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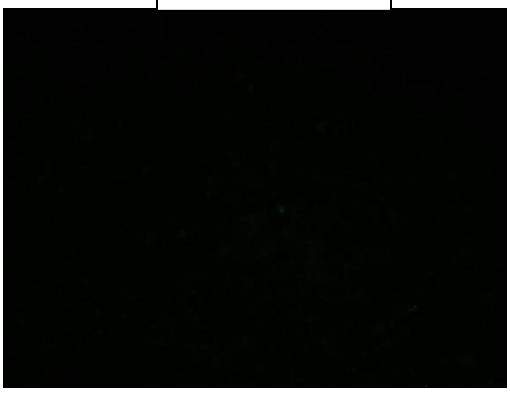

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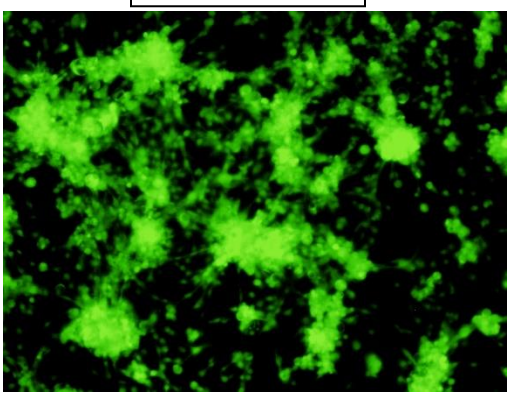

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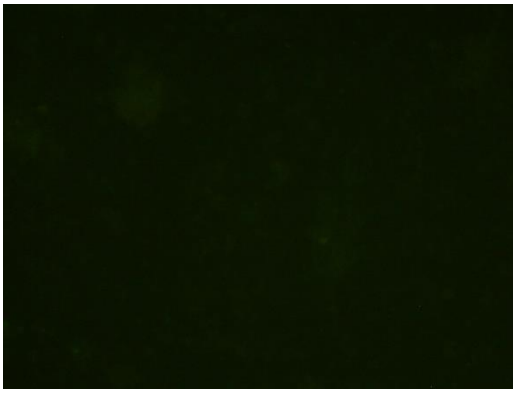

GSR6 set 3 GFP

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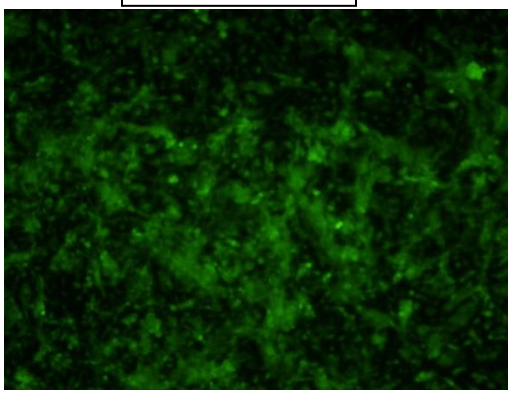

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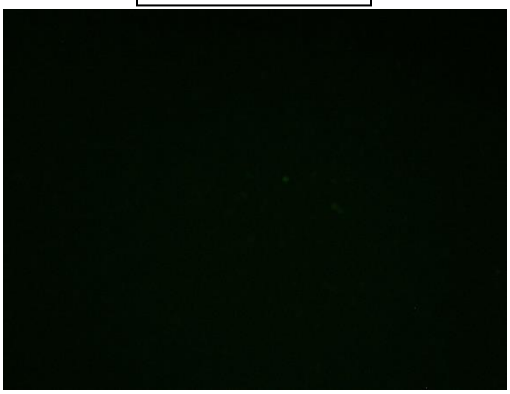

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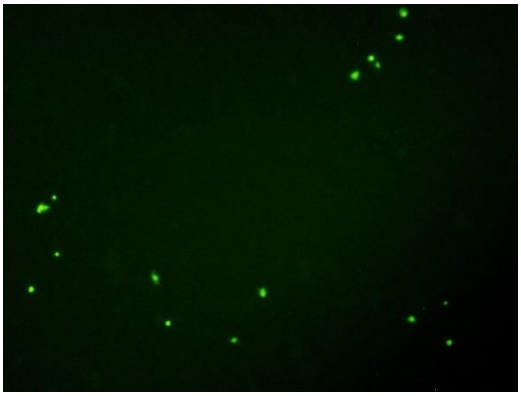

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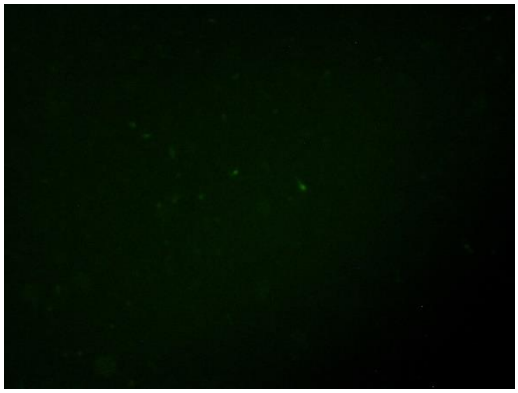

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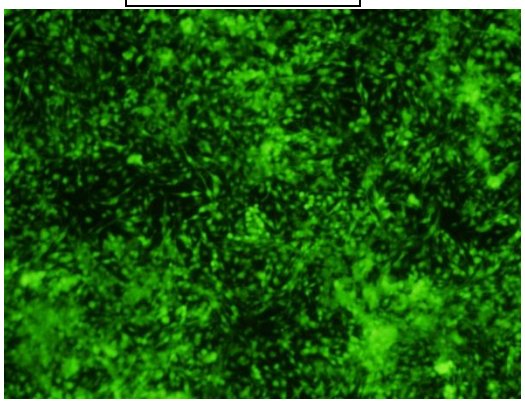

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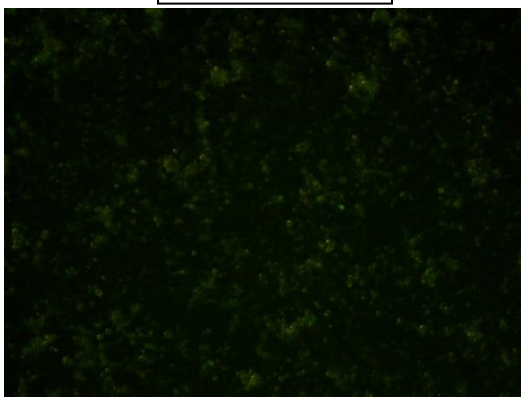

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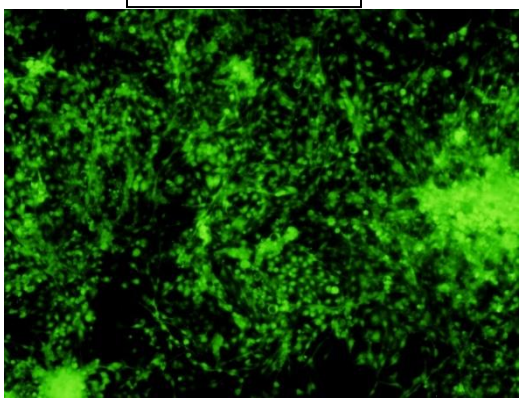

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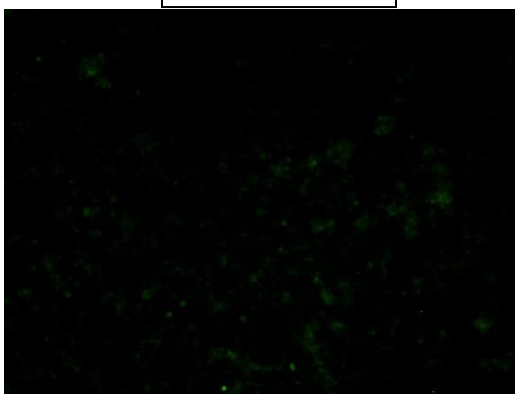

GSR6 Parental Cells

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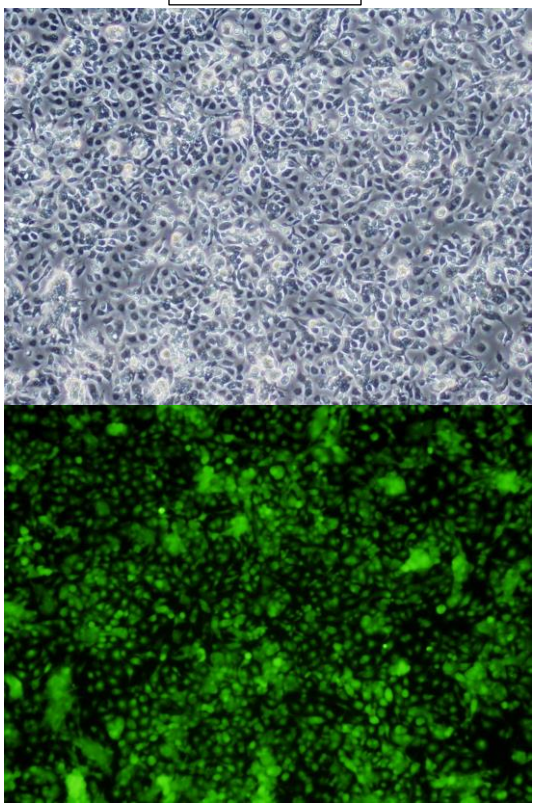

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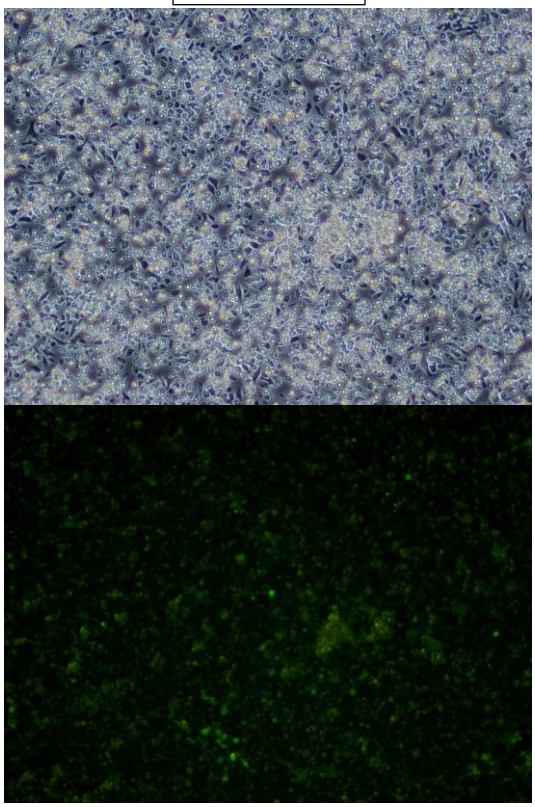

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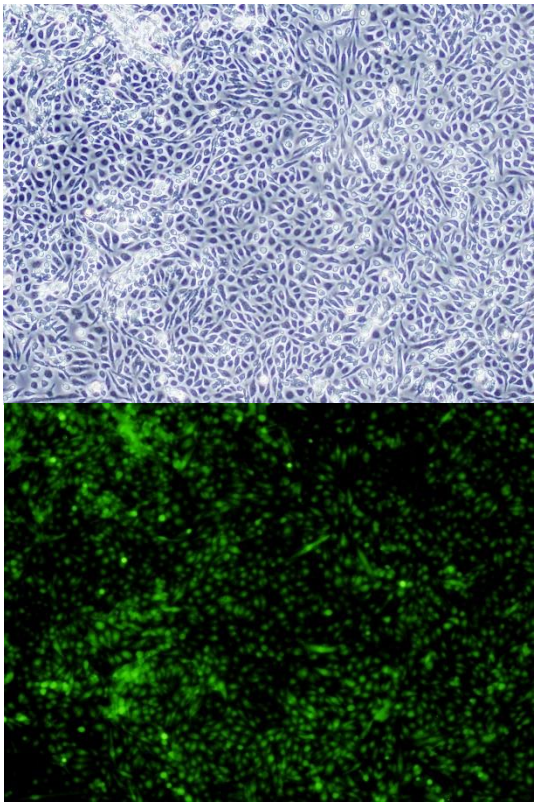

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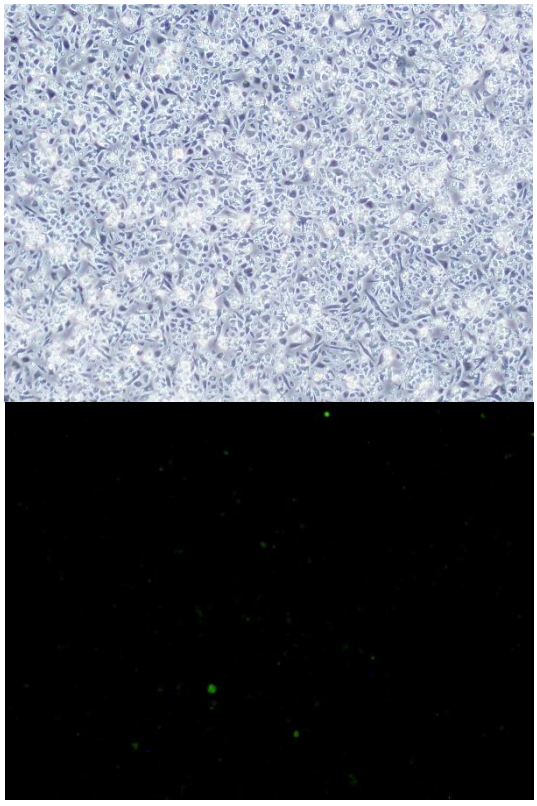

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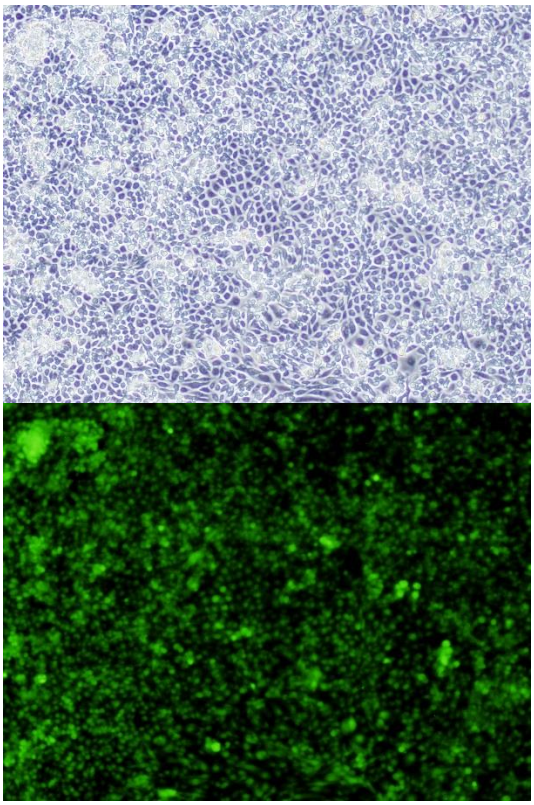

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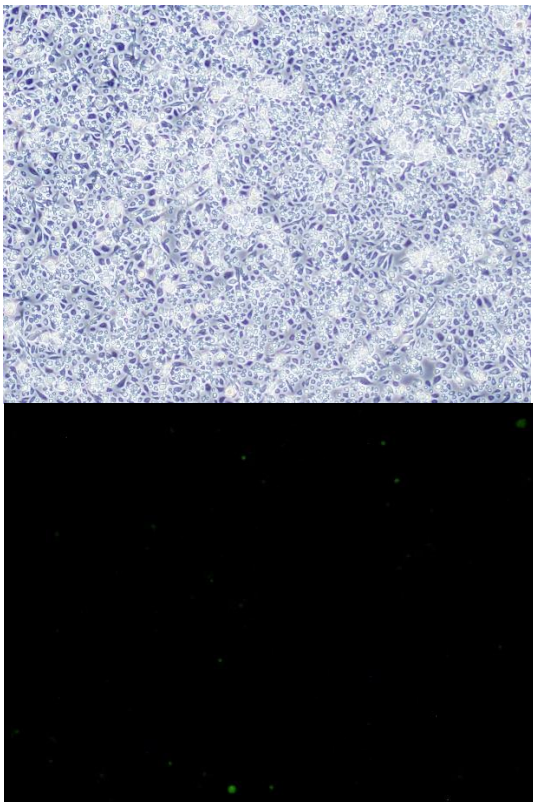

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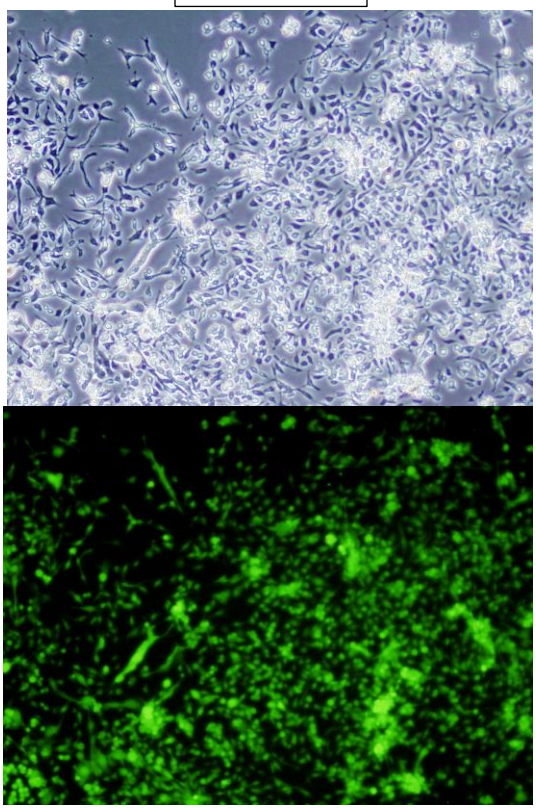

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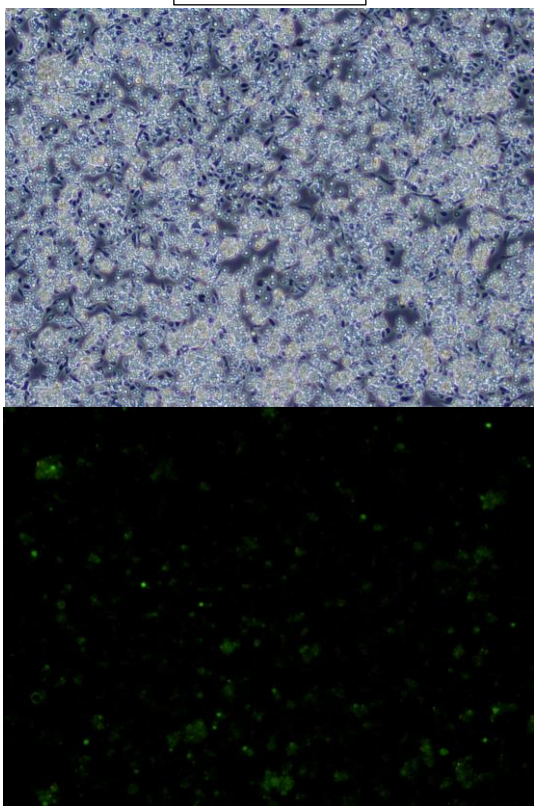

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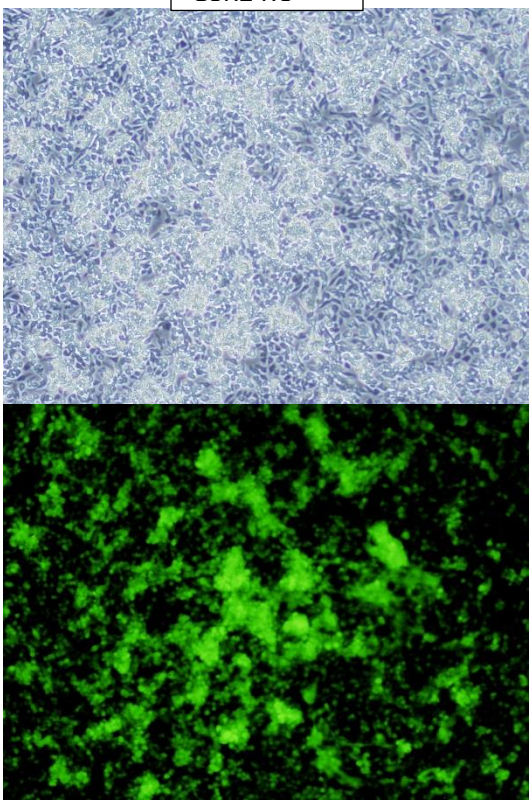

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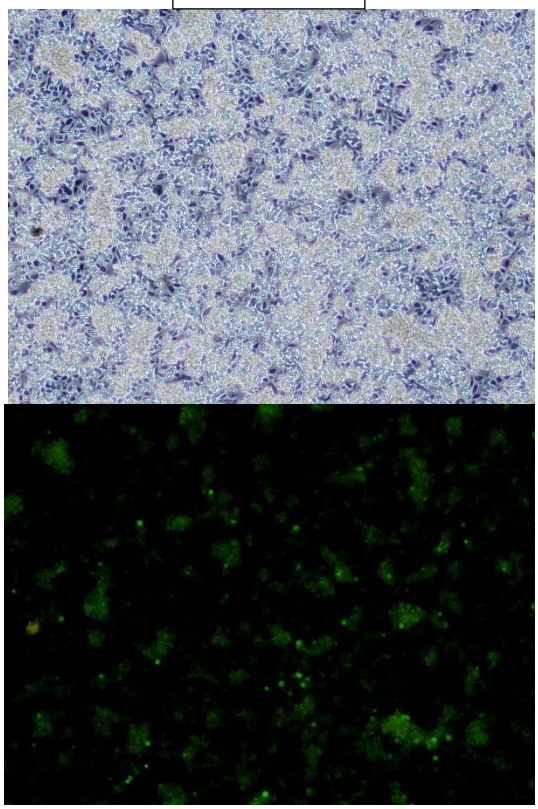

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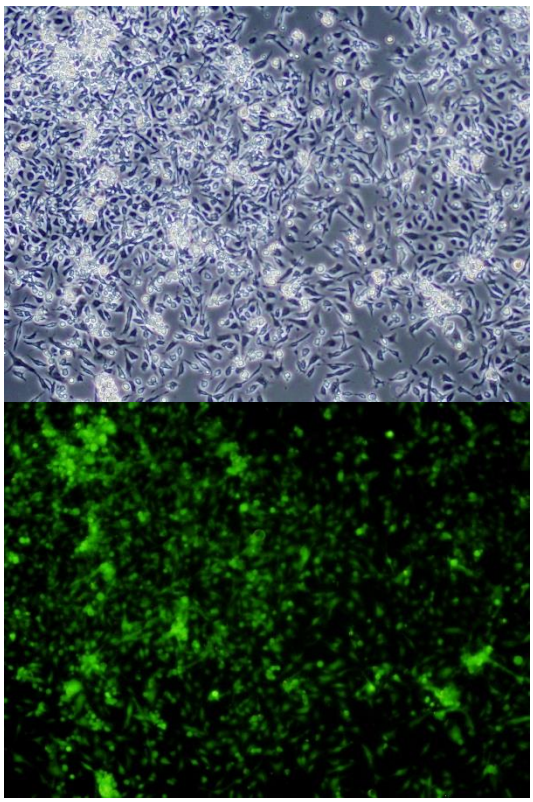

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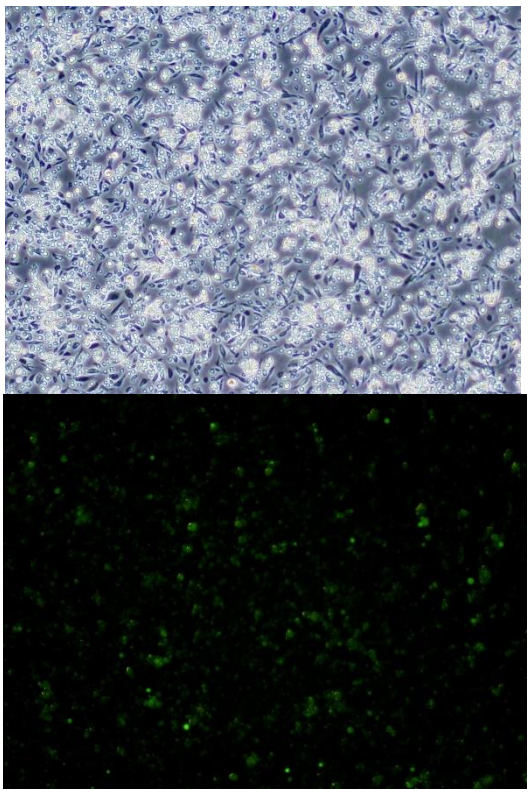

VIII. Appendix – Protocols

Western Blot Protocol

Introduction

General procedure for western blots.

Materials

- 10X Lamelli running buffer (1 L)
  - Tris base - 30.0 g
  - Glycine - 144.0 g
  - SDS - 10 g (recommended to make 10% stock of SDS in distilled water to use.)
  - Disloved in 1000 mL deionized water
  - Store RT
- 10X Western transfer buffer (1 L)
  - Tris base - 30.0 g
  - Glycine - 144.0 g
  - SDS - 1 g (recommended to make 10% stock of SDS in distilled water to use.)
  - Dissolve in 1000 mL deionized water.
  - Store at RT.
  - For working solution, use 1X buffer with 20% methanol. Example Final wokring buffer 800mL 1X buffer wtih 200 mL of Methanol.
  - Store final working buffer at 4 C.
- 4X Separating Gel buffer (100 mL)
  - Tris base - 36.3 g
  - SDS - 8 g (recommended to make 10% stock of SDS in distilled water to use.)
  - Dissolve in 100 mL of water.
  - Adjust pH to 8.8 with 1 N HCl.
  - Adjust volume to 200 mL.
  - Store at RT.
- 4X Stacking Gel buffer (100 mL)
  - Tris base - 1.51 g
  - SDS - 8 g (recommended to make 10% SDS in distilled water to use.)
  - Dissolve in 25 mL of water.
  - Adjust pH with 1 N HCL.
  - Adjust volume to 50 mL.
  - Store at RT.

- 4X Lamelli loading dye (6mL)
  - ~~SDS - 1.2 g~~
  - ~~Glycerol - 4.7 mL~~
  - ~~Tris (0.5 M, pH 6.8) - 1.2 mL~~
  - ~~bromophenol blue - 6 mg~~
  - DTT* (add before use)
  - To 600 uL of loading dye, add 400 uL of 1 M DTT.
- Sample Lysis buffer
  - Tris (1 M, pH 8.0) - 2 mL
  - NaCl (5M solution) - 2.5 mL
  - EDTA (0.5 M, pH 8.0) - 2 mL
  - EGTA (0.5 M, pH 8.0) - 2 mL
  - Triton X-100 - 1 mL
  - Sodium Pyrophosphate - 0.445 gms
  - Sodium ortho Vandate * add just before using 1X Sample lysis buffer
  - Add 10 uL of sodium orthovandate to 990 uL of 1X sample lysis buffer.
  - Add 166 uL of 7X protease inhibitor cocktail (in 1X PBS) to the 1X Sample Lysis buffer.
- 7x Protease Inhibitor (good for 3 months after making)
  - 1.44mL Steril 1x PBS
  - 1 tablet of protease inhibitor
- 10X TBST (1L)
  - Tris - 24.23 gms
  - NaCl 87.66 gms
  - Tween-20 - 10 mL
  - Dissolve in 800 mL of water
  - Adjust pH to 7.5 with 35 N HCL (concentrated). (Takes about 25 mL)
  - Adjust volume to 1000 mL.
  - Store at RT.
- 2X Glycine (500 mL)
  - Glycine - 15g
  - Dissolve in 400 mL of water.
  - Adjust pH to 2.2 with 15 N HCL (takes about 50 mL).
  - Adjust volume to 500 mL.
  - Store at RT.
- 2X SDS-tween (500 mL)

- SDS - 1 g
- Tween-20 - 10 mL
- Dissolve in 500 mL dH2O

- Harsh stripping buffer (20 mL)
  - SDS (10%) - 4 mL
  - Tris HCL (0.5 M, pH 6.8) - 3.2 mL
  - B- mercaptoethanol - 160 uL
  - Make volume up to 20 mL dH2O
  - make before use.

Procedure

Protein Sample Preparation

1. Collect 20 uL pellet volume of cells.
2. Add 100 uL lysis buffer to sample.
3. Vortex vigorously for 1 min.
4. (optional) Homogenize with palastic pestle in an eppendoff tube.
5. Spin down at 8000 RPM for 5 mins. at 4 C.
6. Transfer soup to new tube. (transfer 1-5 uL to another tube for quantification)
7. (Quantification) mix 200 uL NFW and 800 1X bradford to each quantification sample.
8. Add 30 uL of 4x loading dye. (don't forget to add 400 uL DTT)
9. Vortex thoroughly for 1 min.
10. Boil for 15 mins in boiling water.

00:15:00

11. Cool at room temperature for 10 mins. *Turn off hotplate.
12. Spin down for 5 mins at 12000 RPM at room temperature.
13. Collect the bottom sample avoiding top lipid layer.

14. Store the proteins at -80 C.

Gel Preparation

15. Cast gel by pouring sparating gel solution until half an inch below the comb.

Separating gel mix

	A	B	C	D	E
1	Reagent	7.5%	10%	12%	15%
2	Distilled water	1.47 mL	1.25 mL	1.08 mL	0.82 mL
3	4x Separting Buffer	1.37 mL	1.37 mL	1.37 mL	1.37 mL
4	Acrylamid mix	0.65 mL	0.87 mL	1.05 mL	1.31 mL
5	TEMED	3.5 uL	3.5 uL	3.5 uL	3.5 uL
6	10% APS	35 uL	35 uL	35 uL	35 uL

16. Add a layer of 100% ethanol and leave the gel to soldifiy for 15 minutes.

17. Drain off ethanol if needed and make stacking gel mix (1.5 mL)

Stacking gel mix

	A	B
1	Reagent	4%
2	Distilled water	0.98 mL
3	4x Stacking buffer	0.37 mL
4	Acrylamide mix	0.15 mL
5	TEMED	1.5 uL
6	10% APS	15 uL

18. Pour immediately to fill up to the small glass plate and immediately place the comb. Allow the gel to polymerize for 15 minutes. (After may store at 4 C wrapped in paper towel soaked in 1x TBST and saran wrap.)

19. Remove the comb; wash once with distilled water and set up running gel apparatus. Clean wells if needed.

20. Load samples and run.

Transfer

21. Membrane preparation

    Cut membrane to appropriate size (leave 1 cm more on each side greater than the size of the gel. Typical biorad mini gels dimensions are 9.5 cm X 8.5 cm.) Retain the stacking gel if possible. Handle with only forceps. Do not mark membrane, do not puncture membrane.
    Rinse the membrane in methanol for 30 seconds.
    Transfer the membrane to distilled water. Dab the membrane with the tongs until the membrane completely sinks in the water. May take a few minutes.
    Transfer the membrane to 1x transfer buffer and again dab the membrane until it sinks.

22. Pre-soak blotting papers (cut according the gel size) and the sponges in 1x Transfer buffer.

23. make a sandwich of the gel and membrane as follows

    Black side of the cassette, 1 sponge, 3 blotting papers, gel membrane, 3 blotting papers, 1 sponge, white side of the cassette.
    make sure to place the black side of the cassette to face the black side of the transfer apparatus.

24. Transfer in Biorad protein transfer apparatus at 25 V overnight at 4 C or 2 hours at 65 V at RT. (Properly make 1x Transfer buffer: 100 mL 10x Transfer buffer, 200 mL methanol, 700 mL dH2O) Transfer buffer reusable a couple of times.

25. Assess transfer of ladder bands on the blot.

Optional: Transfer assessment by Ponceau S

26. Rinse two times with 1X TBST

27. Rinse the membrane once with 5% acetic acid solution.

28. Wash the membrane with 5% acetic acid solution for 5 minutes.

29. Incubate for 10 minutes with Ponceau S solution. Cover the plastic container with aluminum foil or an opaque box.

30. Drain the Ponceau S solution back to its container and wash the membrane with distilled water.

31. Wash the blot until protein bands become apparent.

Blocking

32. Incubate the membrane with blocking solution enough to submerge the entire blot, for 1 hour at RT or overnight at 4 C.

33. Discard the blocking solution, add the primary antibody, and incubate overnight at 4 C with nutation or for 1-3 hour at RT.

34. Wash three times, for 10 minutes (20 minutes for strong antibodies) at RT with 1x TBST.

35. Incubate the membrane with secondary antibody appropriately diluted in TBST, for 1 hour at RT.

36. Wash three times, for 10 minutes (20 minutes for strong antibodies) at RT with 1x TBST.

Primary antibodies

	A	B	C	D	E	F	G	H
1	Target	dilution	Blocked in	storage	Secondary	2ndary dilution	sizing kDa	
2	Ras	1:2500	nf milk	4	Mouse	1:2500	21	Mouse generally 1:5000
3	Tubulin	1:5000	nf milk	-20	Mouse	1:5000	55	rabbit genereally 1:2000
4	GFP	1:10000	nf milk	-20	Rabbit	1:5000	28	
5	dpERK	1:1000	nf milk	-20	Rabbit	1:1000	42, 44	
6	Erk	1:2500	nf milk	4	Rabbit	1:5000	42, 44	
7	Akt	1:1000	BSA	-20	Rabbit?		60	
8	pAkt (ser 505)	1:1000	BSA	-20	Rabbit?		65	
9	pAkt (ser473)	1:1000	BSA		Rabbit?		60	
10	JNK	1:1000	BSA	4			46, 54	
11	pJNK	1:1000	BSA	-20			46, 54	
12	P38	1:1000	BSA	4	Goat	1:1000	40	
13	pP38	1:1000	BSA	4	Rabbit	1:2000	43	
14	AMPK	1:1000	BSA	-20	rabbit	1:5000	62	
15	pAMPK	1:1000	BSA	-20	Rabbit	1:2000	62	

Chemiluminescent Substrate

37. Mix 1 mL of solution A and Solution B of the chemiluminescent kit ot make the Staining solution.

38. Lay down a Saran wrap on a piece of glass and smoothen it out.

39. Add 1 mL staining solution on the Saran wrap.

40. Gently place the membrane with the 'Protein side' facing the staining solution on the glass place, and incubate 5-30 minutes at RT in the dark.

41. Drain off all staining solution and wrap the membrane in a new Saran wrap.

42. Use dark room and x-ray films to visualize the staining.

Stripping the Membrane

43. Immediately after getting the desired results, remove the membrane from the Saran wrap and palce in a containr with 1X TBST,
44. Rinse the membrane 2 times with 1X TBST.
45. Wash the membrane 3 times with 1 X TBST for 10 mins each at RT.
46. Follow stripping options below

Option A (mild stripping) (Refer to antibody chart for making the choice)

47. mix equal volume of 2X Glycine buffer and 2X SDS & Tween-20 buffer (prepare about 20 mL for each 10 cm^2 blot).
48. add 10 mL (or volume necessary to submerge the blot) and rise the TBST off.
49. Add 10 mL and shake the blot at RT for 15 mins.
50. Rinse 3 times with 1X TBST.
51. Wash 3 time with 1X TBST for 10 mins each, at RT.
52. Block with blocking buffer for 1 hour.
53. (optional: Check for background HRP signal using Chemiluminescent detection kit.)
54. Proceed with primary antibody incubation.

Option B (harsh stripping) (Refer to antibody chart for making the choice) (use saran wrap and do in fume hood)

55. Heat about 20 mL harsh stripping buffer to 50 C (Works well when done in a 50 mL conical tube).
56. Add 10 mL of the stripping buffer to the membrane and rinse wash for 5 mins at 50 C.
57. Remove the buffer, and add rest of the 10 mL to the membrane and wash the membrane at 50 C for 30-45 mins.
58. Rinse 3 times with TBST at RT.
59. Wash 5 times with TBST for 10 mins each at RT. (Repeat wash if the smell of B- mercaptoethanol linger).

60. Wash 2 times with TBST for 30 minutes at RT.

61. Block with blocking buffer for 1 hour at RT.

62. (optional: Check for background HRP signal using Chemiluminescent detection kit.)

63. Proceed with primary antibody incubation.